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Determining Ideal Swab Type for Collection of the Microbiome for Forensic Identification Purposes

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Introduction

In recent years, forensic scientists have begun looking at the microbiome as a new possible human identification method. The microbiome is made up of all the microorganisms living on or in the human body.[1] It is believed that it may be possible to use the microbiome as a unique identifier, to link cohabitating individuals, or even to connect a person with a specific location. In order to study the microbiome, we must first be able to successfully collect it, and then, release it from that collection tool for examination. This may sound simple, but this area of research is so new, even the best method of collection has not yet been determined. Due to the difference in organisms and size of organisms, it is possible that the ideal swab type for this purpose will differ from the ideal swab type for collection of other biological materials and DNA. Therefore, this research focuses on finding the optimal swab type for collection and analysis of the microbiome. Here, a bacterium, *Proteus mirabilis*, will be deposited onto each of four different swab types (traditional cotton, nylon flocked, superfine dental applicators, and Luna dissolvable). Then, extraction of the bacterial DNA will be performed for all swabs, followed by quantitation using real-time PCR, which allowed for determination of absolute microbial DNA recovery and comparison of yields across the four swab types.

This research was begun by another student. The remainder of the research was postponed due to the COVID-19 pandemic and will be completed at a later time.

The Microbiome

As previously stated, the microbiome is made up of all the microorganisms living on or in the human body.[1] Scientists first became aware of the fact that microorganisms might be a part of the normal human flora in the mid-1880s when Austrian pediatrician Theodor Escherich observed a bacterium that would later be known as *Escherichia coli* within the gut flora of healthy infants and infants affected by diarrheal diseases.[2] Escherich was able to isolate this bacteria using his own anaerobic culturing methods and determined that the bacterium was gram-negative using Hans Christian Gram's newly developed gram staining technique.[3] Throughout the rest of the 1880s and the 20th century, various other organisms were isolated and determined to be part of the normal human flora. In 2001, Joshua Lederberg coined the term 'microbiome' for this group of microorganisms.[4]

Also in 2001, the complete human genome sequence was published by the International Human Genome Sequencing Consortium. This was a huge achievement for the field of Biology, but multiple scientists argued that there was more to be done. In a 2001 paper, Relman and Falkow described the need for a 'second human genome project' of sorts. This would encompass a full inventory of the microbial genes and genomes of the four main sites of colonization on the body: mouth, gut, vagina and skin.[5] In a later 2002 paper, Relman spoke of achieving this goal

by using random shotgun sequencing procedures.[6] The Human Microbiome Project was the answer to this problem.

The National Institute of Health's Human Microbiome Project (HMP) was established in 2008 with the goals of characterizing the microbiome well enough to be able to understand how different factors like a person's diseases, age, nutritional habits, medication and environment impact the microbiome as well as the relationship between the human microbiome and human diseases.[5] In its first phase, the HMP characterized the microbiomes of 300 individuals from the nasal passages, oral cavity, skin, gastrointestinal tract and urogenital tract using 16S rRNA sequencing. Metagenomic whole genome shotgun sequencing was also used to look into functions and pathways within the human microbiome. Metagenomic whole genome shotgun sequencing involves randomly breaking the DNA into random fragments to be sequenced separately. Then, the fragments can be put back together based on overlapping in the genetic code. An algorithm can then be used to determine the similarity of the sequence in question and known sequences.[7] The first phase resulted in extensive amounts of data that is publicly available to allow for continuing research.[1] In its second phase, the Integrative Human Microbiome Project (iHMP), researchers are working to collect datasets that include information about not only the microbiome of a person but also other biological properties of the host. Through this, they are working toward HMP's overall goals of establishing the connection between the microbiome and human disease and understanding how different factors impact the microbiome of a person. There are 3 specific projects associated with this phase.

First, a team of researchers are collecting microbiome samples from up to 2,000 pregnant women and their neonates throughout pregnancy, immediately after delivery, and postpartum. Once this data set is established it will be used to investigate what impact the vaginal microbiome has on pregnancy, pregnancy-related complications like preterm birth, and the neonate's microbiome.[8] The second project focuses on Inflammatory Bowel Disease (IBD) which includes both Crohn's Disease and ulcerative colitis. Multiple studies have found links between the composition of the gut microbiome and IBD but none have been able to identify which aspect of the gut microbiome should be targeted for therapy or diagnosis. By studying the dynamics of the gut microbiome of 90 patients for a year, researchers hope to better understand the workings of the gut microbiome in order to more effectively treat and diagnosis IBD in the future.[9] The third project is investigating the links between the microbiome and Type 2 Diabetes. It has already been shown that there are differences in the gut microbiome between diabetics and non-diabetics and that it is possible to lower the glucose levels in mice by altering their gut microbiome. To more fully examine this relationship, researchers are creating a database of samples from 100 individuals determined to be at risk for diabetes at at least 27 different time points. Notice how all three of these projects are jumping off from the "normal" set of microbiome data that was collected in HMP's first phase to now look at the impact of a "not normal" microbiome on human diseases and conditions.[10]

Locations of Microbiome

The first stage of The Human Microbiome Project focused on collection of the microbiome from five different sites: oral cavity, nasal passages, skin, gastrointestinal tract and urogenital tract.

Oral cavity

Bacteria living within the oral cavity were first observed by Anthoni van Leeuwenhoek in the 1680s. The story goes that Leeuwenhoek was very proud of his strict teeth cleaning regimen but when observing his teeth with a magnifying glass one day, to his surprise, he noticed white material between some of the teeth that he described as the thickness of batter. When he looked at the material, which we now know as plaque, under his newly invented microscope, he was able to observe "many very small living animals, which moved very prettily." Leeuwenhoek went on to test the effects of different lifestyle factors on these small living animals that were in reality bacteria. He was able to observe bacteria in the plaque of people known to clean their teeth often, people who rarely cleaned their teeth, people who never drank or smoked, and people who drank and smoked frequently.[11]

Today, the oral microbiome continues to be studied. The mouth provides two places for microorganisms to thrive: the teeth and the oral mucosa. Microorganisms are able to thrive because of the ideal environment the oral cavity provides. The mouth stays at about 37°C with minimal changes, maintains an ideal pH of around 7, and the bacteria are able to stay hydrated and collect nutrients from food intake. Because of the ease of collection, the oral microbiome has been studied extensively. Bacteria from 28 species have been determined to make up the core oral microbiome that remains relatively stable between healthy individuals and bacteria from 157 other genera were observed and determined to be a part of the variable oral microbiome which varies between person to person dependent on things like lifestyle and disease. Of course, the microbiome is not made up of just bacteria. Protozoa, 85 different fungal genera, and different viruses were also observed.[12]

Nasal Passages

When humans breathe in, the air is exposed to all of the microbes within their nasal passages. The human nose is made up of multiple different parts including the middle meatus, sphenoethmoidal recesses, inferior turbinate, and anterior nares. Some of these areas have different cell types and different environments which could potentially allow for habitation by different types of bacteria.[13]

One 2013 study compared the levels of diversity between 3 different areas of the nose: the anterior naris, middle meatus and sphenoethmoidal recess. They found that even while the middle meatus and sphenoethmoidal recess differ significantly in location in the nasal cavity, there was no appreciable difference in their associated microbial communities. These sites are both lined with ciliated pseudostratified columnar epithelium. A different study found that the most common microorganisms for the middle meatus are *Staphylococcus aureus*,

Staphylococcus epidermidis, and *Propionibacterium acnes*.[14] The 2013 study found, however, that the anterior naris, which is lined with nonkeratinized squamous epithelium and sebum producing sebaceous glands, had consistently lower levels of diversity. The study found that the communities of the middle meatus and sphenoethmoidal recess were nearly identical while the anterior naris possessed more Actinobacteria and Firmicutes and less Proteobacteria.[15]

Gastrointestinal Tract

The gastrointestinal microbiome is composed of all of the microorganisms within the gastrointestinal tract. This microbiome site is vast with tens of trillions of microorganisms present. It includes at least 1,000 different species of known bacteria and the majority of those bacteria belong to either the *Firmicutes* or *Bacteroidetes* phyla.[16] The normal microbiome of the gastrointestinal tract can have a symbiotic relationship with the gastrointestinal tract by helping to break down food for energy, synthesizing vitamins and amino acids, and providing a barrier against potential pathogens.[17] People with diseases like irritable bowel syndrome, type 1 diabetes, and some cancers have been shown to have differing gastrointestinal microbiomes, but it is unclear whether the disease caused the changes in the microbiome or whether the disease was caused by changes in the microbiome.

Urogenital Tract

The urogenital tract includes the urinary system and the reproductive system. The human bladder is generally sterile, and the flushing action of the system keeps the ureters and bladder usually free of microbes. The majority of the urogenital tract microbiota is located within the distal urethra for men and the distal urethra and the vagina for women. Bacterial species within the urethra for both sexes are generally consistent with species typical of the skin microbiome. The vaginal bacteria species; however, are more complex. The vagina microbiome works to protect against infections and diseases. To do this, many women have lactic acid producing bacteria that generate an acidic environment. This could be *Lactobacillus*, *Leptotrichia*, *Megasphaera*, or *Atopobium vaginae.* Changes in lactic acid production and the pH of the environment can lead to bacterial overgrowth causing bacterial vaginosis or fungal overgrowth causing yeast infections.[18]

Skin

The skin is a large organ that covers the majority of the human body and comes in contact with various surfaces. For instance, human hands come in contact with doorknobs and other people's hands, human feet come in contact with the ground outside and the floor of the shower, and human foreheads come into contact with cosmetics and acne treatments. Because these skin sites experience such different conditions, the bacterial communities on their surfaces can also vary, making it difficult to define one complete, core microbiome for the entirety of the skin. However, research has been able to identify the types of bacteria that are most abundant at different skin locations.

One 2010 study collected samples from 10 healthy individuals at 20 different skin sites. These skin sites were divided into either sebaceous, dry or moist. Sebaceous included sites like between the eyebrows and next to the nose. Dry included sites included the forearm and buttocks. Moist sites included the nostrils, armpit, and in between the ring and middle fingers. Researchers identified that *Propionibacteria* genus and *Staphylococci* genus were most abundant in sebaceous sites. While *Staphylococci* were also represented in moist sites, *Corynebacteria* species predominated. Bacteria from the class β-Proteobacteria and Flavobacteriales order were the most prevalent bacteria in dry sites.[19]

Like many of the other microbiome sites, a disruption in the normal flora of the skin microbiome has been shown to have a connection to human disease. Multiple studies have attempted to identify the one skin microbe that could be the definitive cause of rosacea. Rosacea is a common skin condition that can cause visible blood vessels and redness on a person's skin. The most common sufferers are middle aged white women who have fair skin but anyone can develop it.[20] The attempts to identify one skin microbe as the cause have not been successful, but one study was able to identify a possible connection between the skin microbiome as a whole and rosacea. In normal skin, the microbiome will activate toll-like receptors (TLRs) as part of the inflammation response. A 2010 study was able to identify an abnormally high activation rate of TLRs in rosacea sufferers that could explain the inflammation and sensitivity that is characteristic of the disease. It is possible that the skin microbiome, as a whole, of rosacea sufferers is activating the TLRs at an abnormally high rate.[21]

Interestingly, rosacea's link to the microbiome does not stop there. A 2008 study found a potential link between the gastrointestinal microbiome and rosacea on the skin. In the study, 113 adults suffering from rosacea and 60 healthy individuals were tested for the presence of small intestinal bacterial overgrowth (SIBO). Those that tested positive for SIBO were given either rifaximin therapy or a placebo for 10 days. One month after the therapy ended, the patients were assessed by a dermatologist. Firstly, patients suffering from rosacea had a higher initial prevalence of SIBO than non-sufferers, with 53 of 113 of sufferers testing positive for SIBO and only 3 of 60 non-sufferers testing positive for SIBO. Of the subjects who received treatment, 20 patient's rosacea lesions cleared and 6 improved greatly. Of the subjects who did not receive treatment, 18 experienced no change in their rosacea and 2 experienced worsened rosacea. When the sufferers who originally were given the placebo were but on rifaximin, SIBO was successfully eradicated in 17 of 20 cases. Of the 17, 15 experienced complete clearage of their rosacea. This study shows that while the microbiomes of different human body sites can vary greatly in their composition and environment, they can also be interlinked.[22]

As evidenced above, all of the human microbiome sites have their own unique environments and, therefore, host their own unique mixture of microbes. Changes in the normal inhibitors of each site have been shown to have a link to different human diseases. Because of this, there are vast possibilities for future research. Here, this research will focus specifically on the human microbiome's potential forensic usage.

The Microbiome in The Forensic Field

In 2010, through three different experiments, Fierer et al. were able to establish multiple key microbiome characteristics. First, to establish individuality of the microbiome, researchers collected samples from the personal keyboards of known people. Then, swabs were taken from the fingertips of those same known people. When the bacterial DNA was extracted and compared, the bacterial communities from an individual's fingertips and keyboard were "far more similar to each other" than to other individuals. Secondly, to look into the stability of microbial samples during storage, armpit samples were taken from individuals and then stored at -20°C or in an open container at 20°C. After two weeks, they observed that the storage methods did not affect the composition of the microbiome or the ability to resolve the differences between two individual's microbiomes. They also completed a third experiment with the goal of determining if an individual's microbiome can be linked to them at the exclusion of the general public. To do this, researchers compared known samples to a database containing 270 samples including the known sample. In all of the comparisons, the known sample's microbiome was more similar to the known standard than the general population.[23]

In 2012, a team of researchers collected fecal samples from 531 healthy people. These people were all from the Amazonas of Venezuela (Amerindian), rural Malawian or metropolitan areas of the United States. After the DNA was extracted from the samples, the V4 region of the 16S rRNA genes was sequenced as well as the total community of DNA.[24] V4 is one of the variable regions of the 16S rRNA.[25] Researchers were able to get V4-16S rRNA data from 528 of the subjects. They then used UniFrac, a program that measures the difference between microbial communities based on the similarity of branch length in the bacterial tree of life. From this, multiple findings were drawn. Possibly the most forensically relevant finding would that there was a significant difference between the bacterial compositions of the microbiomes of subjects who lived in different countries. The most pronounced separation was between subjects living in the USA and those not living in the USA; however, there were also observable differences between the bacterial compositions of the Malawian and Amerindian gut communities. While a fecal microbiome sample might be less likely to be utilized than a skin or oral microbiome sample in forensics, this information could be helpful at times. For example, if an unidentified victim is found and a fecal sample is present, the fecal sample can be used to give clues about the origin of the victim which may aid in the victim's identification.[24]

In a 2013 paper, researchers collected fecal, oral and skin samples from 159 individuals from 60 families. Subjects included spousal units with children, dogs or both. The V2 region of the 16S rRNA genes were amplified to determine bacterial compositions. V2 is one of the variable regions of the 16S rRNA.[25] UniFrac was again used to determine similarity and difference between sites and families. Researchers determined that cohabitating family members had similar levels of bacterial diversity. Also, cohabitating individuals were found to have bacterial communities that were more similar to each other than to other non-cohabitating subjects. Interestingly, this included the human subjects' similarity to their dog's bacterial communities. Notably, these results were strongest for skin microbiome samples. This could be

used in the forensic field to link potential victim to their family members or people they live with.[26]

In 2015, researchers investigated the ability of 16S rRNA sequencing as an analysis method. Sequencing just the 16S rRNA gene has the potential to save laboratories time and money. The 16S is a part of the ribosome that will bind to the coding region of DNA strands. Because of this, the sequence remains relatively conserved between most bacteria. However, the sequence of an individual species of bacteria also contains a variable region that can be used to differentiate between species of bacteria. Primers that are designed to be complimentary to the conserved region of the sequence can be used to target and amplify the variable region in qPCR. These sequences can then be compared to databases like GenBank to identify the species or genus of a bacterium.[27] To test the effectiveness of this method, researchers collected a swab of the bacteria on a communal computer and two known samples from users of the computer. Strains identified through 16S rRNA sequencing were found to be markedly similar to strains identified in the known samples with "Known User #1's" biome differing by only 0.01%.[28]

In a 2016 paper, researchers investigated the stability of the skin microbiome over time. To do this, they collected samples at 17 skin sites from 12 individuals at 3 different time points. Using this data, researchers came to multiple conclusions. First, they concluded that bacterial communities on sebaceous sites, the manubrium and back, were the most stable sites. This was followed by dry sites - notably including the palm, moist sites, and finally foot sites. Possibly the most important conclusion, forensically speaking, came from when researchers looked at the ability of the skin microbiome to remain stable and discriminatory between individuals' over time. To determine this, they looked at the single nucleotide variances (SNVs) of *Propionibacterium acnes,* a very common skin bacterium. SNVs are changes in one nucleotide of the bacteria's DNA sequence. These can be the result of mutations as a strain lives and adapts to being on a person's skin. These SNVs can be discriminatory and result in a person having a strain of *P. acnes* that is unique to them. Researchers were able to determine that regardless of the time interval, a subject shared significantly more SNVs with themselves over time than with any other subjects. Stability of the microbiome overtime is key in its usage in forensic settings. It would not be possible to link a suspect to the microbiome left a crime scene unless the suspects microbiome is still similar enough to the left sample to be considered a match. The findings of this study show that it could be possible to link a person's skin microbiome to a previous sample of their microbiome by looking at the unique SNVs of the bacteria.[29]

In a 2018 interview, Professor Jack Gilbert, director of The Microbiome Center at the University of Chicago, outlines a research project with the National Institute of Justice that is in its very early stages. This research is focused on definitively deciding if the microbiome of a person is unique enough to serve as a forensic 'fingerprint.' This research does not appear to be publicly available at this time. In the interview, Professor Gilbert makes the important point that it is not the different types of bacteria that they believe could differ enough from person to person to be uniquely identifying. Instead, the strain of bacteria a person is carrying could be unique to them due to a person's strain's tendency to mutate and evolve with them overtime.

This idea correlates to the 2015 research described previously where researchers determined that a person's microbiome is stable overtime by looking at the SNVs.[30]

In a 2018 paper, researchers sought to establish whether tape-stripping or swabbing were the idea method for collection of the microbiome. Swabbing is traditionally used widely in forensics for the collection of biological materials, while tape-stripping is more commonly used in the collection of trace evidence like hair, fibers and fingerprints. In the 2018 paper, researcher found that both methods picked up some types of bacteria that the other method did not. However, it was determined that the different bacteria picked up made up a less than 0.5% proportion of the microbiome. Because of this, the study concluded that the two methods performed effectively the same in collection of bacteria. It is important to note that despite the two methods equal ability to pick up effectively the same strains of bacteria, swabbing can still be considered ideal because of the known inhibitory effects that components of tape can have on PCR.[31]

With the microbiome shown to be stable overtime and unique to an individual by several studies, and swabbing determined to be the ideal collection method, we propose to determine which type of swab will produce the highest microbial DNA yield off of the swab, post-collection, for future DNA testing.

Types of swabs

Cotton

The first swab type being explored in this research are Puritan brand cotton swabs. Cotton swabs are traditionally used extensively in biological sample collection; yet they have been shown to have a good absorbance rate but poor elution rate. In a 2014 study, researchers sought to test different modifications to the QIAamp DNA Investigator extraction kit to increase DNA yield from a cotton swab. When they used the provided protocol for the QIAamp DNA Investigator extraction kit, over 50% of the buccal suspension DNA sample and more than 80% of the blood were left within the swab after elution.[32]

Cotton swabs are also notoriously bad for elution of sperm cells. In a 2006 paper, researchers described an enzyme-enhanced way of eluting sperm cells from cotton swabs. To do this, cotton swabs were placed in PCR tubes containing cellulase from *Aspergillus niger* rather than traditional buffer before undergoing a vortexing and incubation process. To test the effectiveness of their new method, researchers compared all of the experimental enzyme samples against samples that were processed normally without the enzymes. For samples dried for 2 days, the samples processed with the enzymes showed a clear cell recovery increase over the normal samples at approximately 27% and 12%, respectively. However, a two-day drying time is not normally realistic for forensic purposes where sexual assault evidence collection kits can sit for months before being processed. At just 30 days of drying, both samples regardless of the enzymes had a percent recovery of less than 2%. Even with this new method, elution of sperm cells from cotton swabs was low.[33]

Flocked

The second swab type that will be included in this research are nylon flocked swabs. These swabs are made with nylon fibers that are positioned perpendicular to the middle of the swab. The swab also does not have an internal mattress core that traditional fiber swabs include. These attributes produce multiple benefits.[34] First, the lack of internal mattress core is believed to help prevent a portion of the sample from becoming strapped within the swab. Additionally, the positioning and material composition of the swab fibers are beneficial. The perpendicular positioning helps to keep the sample near the surface, allowing for easier elution after collection. Similarly, the nylon material allows for a strong capillary-like pull up of the sample. The strong capillary pull up of a large amount of sample by the nylon fibers combine with the swab design's ability to keep the sample near the surface and readily available for elution. Therefore, these swabs should hypothetically collect and then elute more sample than traditional fiber swabs.

Flocked swabs have been shown to outperform cotton swabs in other applications. In a 2018 study researchers asked 119 women between the ages of 21 and 65 years of age to self-collect two vaginal samples. One sample was collected using a cotton swab and one was collected using a flocked swab. A 1:1 randomization was used to determine which sample was collected first.[35] The researchers used flow cytometry to determine the average amount of cells that was collected by each sample. Flow cytometry is done using a flow cytometer which is able to quantify cells and cell properties using a laser. Cells are run by the laser in a single file line and fluorescent and scattered light are quantitated.[36] On average, the number of cells collected per milliliter was 96,726.6 from cotton swabs and 425,544.3 from flocked swabs. So, the flocked swabs yielded approximately 4X as many cells per milliliter as the cotton swabs.[35]

Dental applicators

The third swab type that will be included in this research are dental applicators. Specifically, Plasdent Maxapplicators™ superfine dental applicators will be used. These applicators are traditionally used in dental settings for accurately applying single drops of substances like etchants and sealants.[37] Since they are designed for this precision application, they have a very small surface area. The superfine applicators have a head that is just 1.0 mm in length. In a forensic application, this small surface area could also be advantageous. The idea is that the small surface area will help decrease the possibility of the sample becoming trapped within the swab and remaining in the swab after elution. It appears that there are currently no research studies that have evaluated a dental applicator's ability to collect and release biological samples.

Dissolvable Swabs

This research is focused on determining which swab type produces the best microbial DNA output. To achieve the highest microbial DNA output, the swab must be able to effectively collect the sample and then efficiently release it from the substrate. With this idea in mind, the company Luna designed a swab that entirely dissolves in solution. Hypothetically, if the swab no longer exists, the none of the sample can be left behind in the swab.

The Luna brand dissolvable swabs are made out of cellulose acetate. Cellulose acetate is just 0.2 micrometers in diameter, while typical cotton and rayon fibers are 20 micrometers in diameter. This allows for Luna swabs to a have a 100X greater surface area to assist in effective collection of samples. In one 2017 study, Luna evaluated the performance of their swabs against other traditional swab types: popule, cotton, rayon, and flocked.[38] Popule swabs are self-saturating swabs that contain a solvent within the swab that can be distributed onto the swab head by breaking a seal. The solvent typically includes isopropyl alcohol and water but custom popule swabs can be designed with different solvents.[39] These five swab types were used to absorb biological materials off of unspecified surfaces. In this study, researchers did not take advantage of the swabs dissolving ability. Instead, all of the swabs were put through a traditional DNA extraction method that was not conducive to the swab dissolving. The most DNA was extracted from the undissolved Luna swab, followed by flocked, rayon, cotton, and, finally, popule swabs. Luna credits these results to the previously described cellulose acetate make-up of the dissolvable swabs. These cellulose acetate fibers were able to absorb a large amount of sample while their small fibers prevented the entanglement and entrapment of the sample.[38]

The cellular acetate material of Luna swabs is designed to dissolve in specific chemicals but not when in contact with common liquids that might be found within a house or business. The swabs do dissolve in chaotropic salt solutions which are often a common component of normal DNA extraction kits. Some solvents that Luna swabs will dissolve in include: benzene, toluene, chloroform, and guanidinium thiocyanate, which is a component of several forensic solid-phase extraction kits and the Thermofisher MagMAX Total Nucleic Acid Isolation Kit (ThermoFisher Scientific, MA).

Due to the dissolving nature of the Luna swabs, they cannot be put through the same manipulation procedure that the other swabs are put through. Instead, the swabs will be dissolved in the guanidinium thiocyanate containing component of the MagMAX Total Nucleic Acid Isolation Kit, and then the DNA will be extracted using magnetic bead-based technology. Magnetic bead-based extractions are based on the ability of paramagnetic beads to selectively bind or release DNA based on the solution conditions. First, typically polyethylene glycol (PEG) is used to produce a salt concentration of between 0.5M and 5.0M. In these conditions, the negatively charged DNA will reversibly bind to the magnetic beads. Then, an external magnetic force is used to pull the beads and DNA to the side of the tube. This allows for multiple washes to be performed to eliminate proteins and other contaminants. After all of the undesired material has been separated from the DNA and beads, the DNA is eluted from the beads using an elution buffer that has a salt concentration below the optimal range. This causes the DNA to release from the beads into the buffer solution. From there, the DNA can be amplified through qPCR.[40]

Quantitative Polymerase Chain Reaction

The polymerase chain reaction was invented by Kary Mullis in the early 1980s. This process allows scientists to amplify a small amount of DNA to create a larger amount of product to analyze and work with. This happens in 3 steps: denaturation, amplification, and extension. First, in denaturation, the DNA is heated to approximately 95°C. This causes the hydrogen bonds between the complementary strands of DNA to break. Once the strands are separated, the DNA

is cooled down to approximately 50-65°C. This allows for the complementary primers, which define the region of the DNA to be copied, to anneal to the strands. Finally, the DNA is heated to approximately 72°C to allow for elongation of the copied DNA strand by a polymerase. DNA polymerase will move along both strands, creating two new strands of DNA.[41] In this manner, the amount of DNA is doubled with every successive round of PCR. When this process was first put into practice, scientists manually transferred the DNA to new temperatures for each cycle and repeatedly added new enzymes. Today, scientists are able to use thermocyclers to automatically take the DNA through multiple PCR cycles without the need for any human intervention.

A quantitative PCR (qPCR) allows for the measuring of the amount of DNA present after every PCR cycle. This is done through including a fluorescent dye within the reaction. In this case, SYBR green was used. SYBR green is an intercalating agent. While it will fluoresce naturally in solution, due to a conformational change when intercalated between the bases of double stranded DNA, its fluorescence will increase by up to 1,000-fold. Therefore, the amount of fluorescence present will be proportional to the amount of double stranded DNA product that has been produced.[42] At the beginning of qPCR, the amount of fluorescence is below threshold, or the level that the instrument is able to read. Once the amount of DNA product has reached a readable level, it will be considered to have passed the 'threshold line.' The cycle number where the product passes this line is referred to as the 'cycle threshold,' or C_T value. The C_T value is dependent on the starting amount of DNA. A larger DNA input amount will require fewer cycles to produce enough double stranded DNA product for fluorescence to reach the cycle threshold. So, high starting DNA amounts will result in lower C_T values. Similarly, lower starting DNA amounts will require more amplification cycles to produce enough fluorescence to be detectable and will, therefore, have higher C_T values.[42]

The qPCR has three non-temperature dependent phases that are defined by the amount of product being produced at that point. These three stages are the exponential phase, the linear phase, and the plateau phase. After several cycles that allow for the PCR amplification to produce enough fluorescence to surpass background noise, the reaction enters the exponential phase. In this phase, the product is effectively doubling after every cycle, and this is where the reaction will cross the threshold and produce the Ct value. This rapidly provides new double stranded DNA product for the SYBR green to intercalate with and produce fluorescence. Fluorescence exponentially increases in this phase. Eventually, the reaction slows, as components of the PCR reaction become limiting, and enters the linear phase. Finally, the reaction will enter a plateau phase. This phase can occur as a result of one or more of the necessary components of the reaction being used up. So, during this phase the product production will decrease and eventually cease.[43]

The qPCR Master Mix

Primers

The key factor that makes PCR so powerful is its ability to amplify a specific region of a DNA strand. This specificity is achieved through the use of primers. Primers are short

oligonucleotide sequences that will anneal to the DNA strand and create a starting point for DNA polymerase to begin copying the strand. Primers are designed to be complementary to sequences slightly downstream or upstream from the actual ideal starting point, dependent on the strand. These sequences tend to be relatively conserved between organisms, which aids scientists in the design of primers. In order to completely define the correct region, both a forward and reverse primer is used. The forward primer will anneal to the start codon of the anti-sense strand and the reverse primer will anneal to the stop codon of the sense strand.[44] Together, both of these primers will fully design the goal region. Poor primer design can reduce reaction efficiency, meaning the product will not actually double after each cycle. The primer needs to anneal to the correct region and provide a starting point for extension in order for new product to be created.

iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, CA)

This Supermix contains multiple components that are necessary for the qPCR process. First, it contains Taq DNA polymerase. The qPCR process requires a DNA polymerase to move along the original strands, copying them during the extension step, in order to create the new strands. While some polymerases are inactivated at high temperatures, in 1966 Thomas D. Brock isolated a thermophile, *Thermus aquaticus,* from the hot springs at Yellowstone National Park.[45] From *T. aquaticus,* a polymerase was isolated that would be known as *Taq* polymerase. Because of its native environment, *Taq* polymerase is able to work efficiently at high temperatures, like the temperatures used in PCR. *Taq* will start where the primers are annealed and will move along the DNA strand, 'reading' it, and adding deoxynucleotide triphosphates (dNTPs) to the newly forming strands during the extension phase.[46]

Next, Supermix contains $MgCl_2$. Magnesium (Mg^{+2}) levels must be very closely monitored to successfully work with DNA. Mg^{+2} is a cofactor for nucleases. Nucleases are enzymes that breaks down DNA. During DNA extraction, Ethylenediaminetetraacetic acid (EDTA) is used to bind up Mg^{+2}, preventing it from interacting with nucleases, and, therefore, preventing the activity of the nucleases in the digestion of DNA. Interestingly, Mg^{+2} is also a cofactor for *Taq* polymerase. The Mg^{+2} binds to the polymerase and catalyzes its action. Here, that means it helps the polymerase more efficiently add dNTPs to the newly growing strand. [47]

Third, the Supermix contains SYBR green I dye. As mentioned previously, SYBR green is a fluorescent dye that will intercalate between the adjacent bases in double stranded DNA. When it comes into contact with the double stranded DNA, its florescence will increase. As more and more double stranded DNA product is created my PCR, the florescence from SYBR green will proportionally increase. This fluorescence will be read by the instrument after each cycle.

Finally, the Supermix contains passive reference dyes like carboxy-X-rhodamine (ROX) and fluorescein. Passive reference dyes produce fluorescence. Their fluorescence is used to normalize fluorescence across the wells of the reaction plate, which helps to compensate for inter-well variations in fluorescence due to differences in the length of the optical path or well volume .[48]

Experiment Procedure

The first two swab types, cotton swabs and nylon flocked swabs, were tested by a separate student. The superfine dental applicators and Luna dissolvable swabs will be tested at a later time.

To begin this research, *Proteus mirabilis* was spotted onto a lysogeny broth (LB) plate. The plates were left to incubate overnight at 37°C. After clear colonies had developed, a colony was taken from the plate, and put into a liquid culture LB broth. The liquid LB culture was left to shake for approximately six hours. After six hours, the liquid was aliquoted into four Eppendorf tubes and stored at -80°C for future use.

In one round of testing, 5 µL of *Proteus mirabilis* was directly pipetted onto the tip of three cotton swabs (Puritan Medical Products, Guilford, ME). 5 µL of *Proteus mirabilis* was also directly pipetted onto three 4NG FLOQSwabs® (Copan Diagnostics, Murrieta, CA). After allowing time for the bacteria to completely sink into the swabs, the entire head of the swabs were removed and transferred into Eppendorf tubes containing 500µL of Tris buffer. Additionally, 5 µL of *Proteus mirabilis* were pipetted directly into three additional Eppendorf tubes containing 500µL of Tris buffer; these were to be used as for a positive manipulation control. Finally, 5 µL of *Proteus mirabilis* was pipetted directly into one Eppendorf tube containing 45µL of Tris; this was for use as the direct amplification/non-manipulation control.

Next, all nine Eppendorf tubes, not including the direct amplification control, underwent a vortexing process to release the bacteria from the swabs. First, the tubes were all vortexed for 5 minutes by an automatic vortexer that was set at speed 8. Next, each tube was individually vortexed manually for 30 seconds. Finally, the tubes were vortexed for 5 more minutes on the automatic vortexer at speed 8. After vortexing, each of the swabs was transferred to a spin basket, and the spin basket and swab were replaced into their corresponding microcentrifuge tubes. The tubes were closed and then centrifuged for 5 minutes at 5,500 rpm. After 5 minutes, the supernatant and spin basket were discarded from each of the tubes, but the swabs were retained and transferred to new spin baskets. Then, 500µL of new Tris was added to each tube, and the swabs in the new spin baskets were replaced in the tubes. The tubes were once again centrifuged for 5 minutes at 5,500 rpm. After this round of centrifugation, the supernatant, spin basket, and swab were all discarded. The remaining cell pellet and liquid were measured using a micropipette. Each pellet was resuspended in enough Tris buffer to leave each tube with 50µL total volume. Now, all samples, including the direct amplification control, were prepared for qPCR analysis.

For qPCR, a triplicate was created from each of the 3 cotton swab tubes, the 3 flocked swab tubes, the 3 positive control with manipulation tubes, the direct amplification positive control, and a non-template control (NTC). There were 33 qPCR reactions total. To create the triplicates, first, a master mix was created using 102.5 µL of H_2O, 10.9µL of each primer, and 155.3 µL of iTaq. After creating the master mix (MM), 27µL of MM was added to the first tube of the NTC triplicate. Then, 3µL of H_2O was also added to the same tube. That was pulse vortexed and centrifuged, and then distributed so that there was 10 µL in each tube. For the other

30 tubes, 27 µL of MM was added to the first tube in each triplicate already containing 3µL of the corresponding template DNA. The tubes were vortexed, spun down, and then distributed so that there was 10 µL in each reaction tube.

After allowing the PCR machine to heat up to 95°C, the samples were loaded and allowed to run for 30 cycles with the following settings:

Forward Primer and Reverse = 10 µL
Final concentration = 350
Number of Reactions = 33
Reaction Volume per Well = 10 µL
Template Volume = 1.0µL
Super Mix Concentration = 2.0X
Excess Reaction Volume = 10%

This process will be repeated for dental applicators and dissolvable swabs with alterations to the procedure for the dissolvable swabs. The dissolvable swabs will be processed according to the provided MagMAX™ Total Nucleic Acid Isolation Kit procedure. The swabs will be dissolved in a guanidinium thiocyanate-based solution. Zirconia beads are used to disrupt the sample and release the nucleic acids. Then, the samples will be diluted with isopropanol and paramagnetic beads will be added. The paramagnetic beads will bind the DNA. The beads and DNA will be pulled to the side by a magnet and washed repeatedly to remove proteins and contaminants. Once all of the contaminants have been removed, the DNA will be eluted off of the beads into a small volume of low-salt EDTA buffer. These samples will then be processed and run on qPCR as described above.[49]

Data Analysis Procedure

Once all of the swab's samples have been amplified by qPCR, their C_T values will be compared to a standard curve that includes C_T values of samples with a known DNA concentration. From the known standards, a linear regression line can be created. The unknown sample's C_T values will be plugged into the equation of the standard curve to determine the DNA concentration of the unknown samples.

After all DNA concentrations have been determined, the concentrations of all swab types will be compared to determine which yielded the highest microbial DNA recovery. To do this, the direct amplification control will be used as an approximation of a theoretical 100% DNA yield. The quantities of DNA obtained from each swab type will be averaged to obtain the overall yield for that swab type. The average yield for each swab will be divided by the yield from the direct amplification control to determine the percent microbial DNA recovery. Both the average yield across all of the swab types and the percent recovery will be compared. This will be done using an analysis of variance statistical test (ANOVA). The ANOVA will be used to determine if the potential variance between swab types is statistically significant. If a statistically significant variance is found, a post-hoc analysis will be performed to determine which of the swab types were statistically different.

Controls

Positive Control with Manipulation

The positive control with manipulation was included with the goal of determining how much microbial DNA is lost during the manipulation process itself rather than from being left in the swab. This control followed the other sample Eppendorf tubes through the entire process with all of the opening and closing of the tubes, transferring of spin baskets, and vortexing.

Direct Amplification/Non-Manipulation Control

The direct amplification control was included to provide a starting or theoretical 100% yield value for the microbial DNA. This control did not undergo any manipulation and did not involve any swabs. The Tris and *P. mirabilis* were combined in the Eppendorf tube and then added directly to the qPCR. The microbial DNA yield from this sample can be compared to the yields from the swabs and manipulation samples to determine the percent yields.

Negative control

There was purposely no substrate negative control included in this experiment. This is because if the results were to indicate that no contamination was present, that result would only apply only to that specific swab. It would not necessarily indicate whether the other swabs used in the experiment were contaminated in anyway.

A non-template control (NTC) was used during qPCR. The NTC contained only 27 µL of master mix and 3 µL of water instead of template DNA. This is used to monitor contamination of the master mix or water used. Since there was no template DNA added, there should be no DNA amplification. If amplification is observed, there is likely contamination.

Proteus mirabilis

The bacteria that will be used in this study is *Proteus mirabilis. P. mirabilis* is a gram-negative bacillus of the Enterobacterales family. This bacterium is known to swarm and create a characteristic bulls-eye effect when cultivated on an agar plate. This pattern is due to the bacterium's ability to repeatedly differentiate from short swimmer cells to long, highly flagellated swimmer cells.[50]

This bacterium was chosen for this research by the student who began this research for two reasons. First, this bacterium was readily available after another researcher in the lab isolated it from a rat that had been used in another study. Second, *P. mirabilis* is most commonly associated with urinary tract infections and nosocomial infections, or infections of those in hospital care. Because of this, a normal healthy person would likely not have this bacterium as a part of their skin microbiome. This could potentially be helpful for a future research project related to this study where researchers are able to sample the microbiome from actual human subjects. This way, they would be able to know that if a percentage of *P.mirabilis* is observed, it very likely came entirely from the test bacterium rather than other bacterium native to the environment. Additionally, since this bacterium is not found on the average person's hand or an

average clean environment, experiments are less likely to be contaminated by outside sources of this bacterium.

Further Research

Due to Bowling Green State University suspending undergraduate research as a result of the COVID-19 pandemic, the remainder of the laboratory experiments involved in this study will have to take place at a later time. This research will help to establish the groundwork for other future studies. Firstly, a time-study could be done. After the swab type with the best microbial DNA yield has been established, researchers could test the swab's ability to absorb and elute a sample after different intervals of time in order to more closely replicate forensic collection conditions, where samples are not usually collected immediately after deposit. Secondly, a surface study could be done. Bacteria could be spotted onto various common household surfaces like tile, laminate, wood and glass and then picked up by the best performing swab. The percent microbial DNA recoveries for each surface could be compared to determine from which surface the swab is able to best pick up the sample. Finally, it would be interesting to expand the described procedure from this study to include a second round of elution. This data would identify whether there is any sample remaining stuck within the swab and if so, if it can be released through a second round of elution.

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