Potential Use of Microbiota as a Forensics Tool to Determine a Post-Mortem Interval

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Abstract

Recent studies have discovered that cadavers have statistically significant time-, organ-, and sex-dependent changes within the thanatomicrobiome and necrobiome. Additionally, researchers have learned that these changes work together to break down dead organic matter. These studies show a successional change in the thanatomicrobiome corresponds to the five stages of decay. In the early fresh stage, microbes indigenous to the once-living body are most dominant. However, as the corpse enters the bloat stage, bacterial groups that are part of the natural gut microbiome spread as tissue liquefies. Next, as oxygen levels deplete within the corpse as it enters advanced decay, there is a proliferation of obligate anaerobes. Finally, as the corpse begins to skeletonize, the microbiome begins to look more like soil bacterial communities. Further research has indicated that these successional changes may be in response to the physical and chemical changes that occur in the decaying corpse. Because of this apparent correlation, researchers suggest that these microbiotas may prove to be useful to forensic scientists seeking to determine a more narrow post mortem interval. This research is very much still in its infancy and has yet to be proven to be effective enough to function as evidence in the criminal justice system. However, future research may expand on the current research gaps and create a more robust system for tracking bacterial development as correlated to the stages of decay.

Introduction

As the human body decays, it goes through five distinct and defined stages: 1) fresh, 2) bloat, 3) active decay, 4) advanced decay, and 5) dry remains. Each stage has different biological processes associated with it; for example, early stages of decomposition include rigor mortis (stiffening of the body) and livor mortis (lividity or pooling of the blood), whereas, later stages include liquefication of soft tissue and the beginnings of skeletonization. The process of decomposition is mainly driven by the activity of insects and microorganisms which themselves are influenced by biotic and abiotic factors in their environment (Damann et al., 2015).

These stages of decay have been studied for decades and today are used by forensic pathologists to determine the post mortem interval (PMI), the length of time between the point

of a person's death and the time of examination by a forensic pathologist (Schmitt et al., 2010). Determining an accurate PMI is important to the investigation of untimely deaths and criminal cases as it can provide insight into the cause of death, aid in the identification of remains, and confirm alibis.

Forensic pathologists consider many factors when assessing a decedent's PMI. As mentioned previously, pathologists frequently consider as significant the stages of decay coupled with autolysis and putrefaction (the release of enzymes that degrade cellular protein and the subsequent breakdown of tissue) (Schmitt et al., 2010). Forensic pathologists may also analyze the generational development of blowfly maggots on the corpse or changes in the color of the body's internal organs (Schmitt et al., 2010). However, all methods for determining an accurate PMI have limitations on them. Biomarkers of decay can be heavily influenced by environmental factors. For example, the process of decay can be sped up or slowed down by nearly fifteen days depending on the ambient temperature. Additionally, maggots--which are often used as biomarkers of decay--are often completely killed off in the winter season (Carter et al, 2015). Therefore it is generally most effective for pathologists to use a combination of many biomarkers to get more accurate results (Debruyn and Hauther, 2017). As such, researchers are developing a new tool for forensic pathologists as a potential biomarker to help determine a narrower window for the PMI: our bodies' own microbiota.

The microbiota communities that work together to decompose a corpse can be divided into two broad categories, the necrobiome and the thanatomicrobiome (Javan et al.,2016). The necrobiome is the community of organisms that decompose the body from the outside, including bacteria, fungi, and insects that live in the soil or are carried in the air. The study of the thanatomicrobiome, a term coined by Gulnaz Javan in 2014, is the community of bacteria that inhabit a human body after death. It is characterized by a successional process where these microbes inhabit, grow, and eventually die within their host body, resulting in a shift in the composition of the entire community as their host continues to decay over time (Javan et al., 2016). Because the thanatomicrobiome is already present inside the corpse, the thanatomicrobiome is generally not as heavily influenced by the same environmental factors that affect the necrobiome. However, the combination of both communities interacting with each other and their changing environment (the decaying host) may be major contributors to decomposition (Javan et al. 2016).

Certain microbes are able to proliferate well after the death of their host due to the rapid change in their environment as the host cells die off and break down in the process of putrefaction. This breakdown of soft tissue floods the microbiome with a wealth of nutrients and chemicals to feed on. However, as these microbes go through their respective life cycles within the corpse, they demonstrate a successional change in the overall microbiome diversity (Javan et al., 2016). Many studies have shown, after standard sequencing results of 16S rRNA gene amplicons for postmortem samples from human and animal cadavers, that there is a statistically significant time-, organ-, and sex-dependent change within the microbiota of cadavers (Javan et al. 2016; 2017; Hyde et al. 2016; Johnson et al. 2016; Bell et al. 2018; Debruyn and Hauther, 2017; Adserias-Garriga et al.

2017; Carter et al. 2015). These studies suggest that the use of the thanatomicrobiome and necrobiome may prove to be useful to forensic scientists in determining a more narrow PMI.

Scientists have been able to track these changes in community diversity and create catalogs that show the most common groups of organisms present at various PMIs. Furthermore, research has also shown that the succession of microbiota is repeatable across replicates for animal carcasses as well. Therefore, it can be concluded that controlled decomposition studies using mammalian carcasses may also provide insight into the decomposition of much larger mammals like humans, and these studies may serve to facilitate further forensic research without the use of human cadavers (Carter et al. 2015; Preiswerk et al. 2018; Lawrence et al. 2019). In this literature review, we will examine the current research and how far scientists have progressed in sequencing these microbiotas of decay.

First, we will look at the similarities and differences that have been analyzed across studies on microbial succession. Second, we will focus on specific studies on biotic and abiotic factors that may influence both the necrobiome and the thanatomicrobiome. Finally, we will examine specific taxonomic groups of microorganisms that may be key biomarker species in determining an accurate PMI.

Successional Changes in Various Microbiota Communities

As the body continues through the five stages of decomposition, many biological and chemical processes rapidly transform the internal environment of the corpse. For example, in the bloat stage, the body begins to liquefy and the overall pH rises (Schmitt et al., 2010), which in turn causes the overall composition of the microbiome to change (Debruyn and Hauther, 2017; Javan et al. 2016). Although over time, the microbiota communities increase in species richness (the number of individual species), the overall diversity actually decreases, indicating a decrease in species evenness (number of individuals of a species present in a population). This change in evenness usually seen in microbiology as a response to environmental disturbances or bacterial blooms (Debruyn and Hauther, 2017). It is likely that the rapidly shifting environment and production of putrefaction byproducts stress some members while others are able to proliferate (Debruyn and Hauther, 2017). This overall decrease in evenness in response to physical changes means that certain species may dominate in numbers over others and, therefore, could be key biomarkers for certain stages of decomposition. For example, a key change in a decaying corpse is the rapid depletion of oxygen that is no longer being pumped throughout the body by a functioning circulatory system. This change has been shown to shift the microbiota from aerobic to anaerobic members that can survive without oxygen (Javan et al., 2016).

In a healthy adult, most internal organs such as the heart, lungs, or liver are essentially sterile (Javan et al. 2016). However, after death, the thanatomicrobiome that is inherently present in the gut is able to spread without restraint from a properly functioning immune system (Javan et al. 2016). Because each organ and anatomical zone of the

body has its own unique function and biological process, the rate of decay and overall microbiome differs in each organ. For example, the gut and intestine typically will begin putrefaction within just a few hours, whereas the uterus or prostate will typically decay last, some days later (Schmitt et al., 2010). This difference may be due to gut bacteria that are already present in the body that give a jumpstart to the decay process in the gut and intestine, as opposed to the more sterile reproductive organs. Some studies have also suggested that muscular tissue makeup could be a factor as well that limits bacterial growth (Schmitt et al., 2010). Furthermore, the abundance of oxygen in the heart and lungs can greatly influence the presence of aerobic bacteria in these areas (Javan et al. 2016, Schmitt et al., 2010). As such, scientists typically will study each zone individually in order to eliminate as many variables as possible. The following sections will examine the results of such studies on the succession of these microbial communities in various anatomical systems of the body.

Intestinal Tract

The gut and intestinal tract are inherently filled with millions of bacterial cells that help a living body digest and ferment nutrients from food. Once the host dies, these bacteria become the starting point for the thanatomicrobiome that decays the body from the inside. Researchers found that there is a time-dependent split in the makeup of the thanatomicrobiome in the gut (Debruyn and Hauther, 2017; Adserias-Garriga et al. 2017; Javan et al. 2016). Research demonstrated that early-stage communities have a higher abundance of the phylum Bacteroidetes and the Firmicutes, whereas late-stage communities have lower diversity than the early microbial communities. Although Firmicutes (in particular Clostridiales) still dominate in these communities, the communities have a reduced abundance of Bacteroidetes. Both Bacteroidetes and the Firmicutes are indigenous to the human gut and aid in the digestion of important macromolecules and proteins (Debruyn and Hauther, 2017).

This shift in the community of the higher presence of Firmicutes and a lesser presence of Bacteroidetes was typically shown to occur at the second stage of decay known as the bloat stage (Debruyn and Hauther, 2017; Adserias-Garriga et al. 2017). In the bloat stage, the corpse will begin to swell, sometimes doubling in size, as gases accumulate as a byproduct of bacterial fermentation caused by the bacteria that are indigenous to the gut microbiome (Schmitt et al 2010). The build-up of gases also pushes the liquid out of the body and signifies the transition from early decomposition to more advanced decay. Debruyn and Hauther suggest that the community shift mid-way through the bloat stage may be caused by many factors, including physical changes due to tissue break down, changes in the chemical environment as putrefaction byproducts (such as ethanol buildup) or competition with other species.

Oral Cavity

The oral cavity inherently has some of the most diverse microbiota communities. Because of this inherent presence, bacterial families including Lactobacillaceae, Staphylococcaceae, Streptococcaceae, and Actinomycetaceae have been shown to be most dominant in the early fresh stage of decomposition (Adserias-Garriga et al. 2017);

as decay progresses, however, these phyla begin to decrease in abundance and the phylum Tenericutes begin to appear (Adserias-Garriga et al. 2017). Tenericutes have been shown to develop in the oral cavity once a corpse has reached the bloat stage. Tenericutes in a living body are normally part of the gastrointestinal microbiome; therefore, their presence in the oral cavity during the bloat stage may be due to fluids being pushed out of the body by the production of gases from other bacteria in a process termed a purge. Furthermore, Tenericutes will typically appear in the oral cavity even if the bloat stage is not physically visible due to environmental factors or the physical build of the corpse (Adserias-Garriga et al. 2017). Other research on the oral cavity found that as the oral cavity decays, Firmicutes like Clostridium *spp.* and other obligate anaerobes like genus Prevotella dominate later in decomposition ((Javan et al., 2016). These two studies also found that the presence of the bacterial families Gammaproteobacteria, Pseudomonadaceae, Alcaligenaceae, and Planococcaceae were much higher during late decomposition (Javan et al., 2016; Adserias-Garriga et al., 2017).

These studies demonstrate a change in the oral cavity thanatomicrobiome from facultative anaerobes (bacteria that can tolerate oxygen such as Staphylococcaceae and Streptococcaceae) which are initially indigenous to the oral cavity, to obligate anaerobes (such as Clostridales, Prevotella *spp.* and Pseudomonadaceae) as the lingering oxygen level decreases over time. A key species biomarker from these studies is the Tenericutes, which seem to proliferate specifically during the bloat stage in the oral cavity, which is an important stage to distinguish the transition from early to late decomposition.

Skeletal System

An important factor to consider when analyzing a corpse is that the typical techniques for estimating PMI require the presence of soft tissue. Therefore, as a corpse decays along into the skeletonized phase, the ability to estimate a PMI becomes more difficult. To address this difficulty, one study analyzed the decomposition of rib bones from a group of human cadavers aged at different ranges of PMI (Damman et al.,2015). This study analyzed the evenness of six different bacterial groups in each bone sample, including Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria, and Chloroflexi. This study found that the youngest samples, which were only partially skeletonized and had a PMI of 27–284 days since death, had the highest proportion of Firmicutes than any other group. Bacteroidetes were the most abundant for the skeletonized sample (PMI of 292–369 days), and the dry remains or oldest group (PMI of 554–1692 days) had the largest proportion of Actinobacteria. This study also found that the bacterial communities of the oldest group were more similar to control soils than were younger groups. The control soils used in this study contained a high abundance of Actinobacteria and Acidobacteria, which are naturally soil-dwelling groups of bacteria. Researchers determined that these two groups of bacteria increase in abundance as the age of the bone samples increased. In earlier stages of decay, these bacteria may not be able to develop well due to increased soil pH and nitrogenous leachate. This study demonstrates the corpse's microbiota community changes from bacteria indigenous to the human gut (Firmicutes and Bacteroidetes) to bacteria that is more common in soils (Actinobacteria and Acidobacteria) (Damman et al.,2015).

To summarize, all of these studies on various zones of a corpse show a successive change in the thanatomicrobiome that is directly influenced by the changes in their decaying host. The studies also suggest change within the corpse can be brought about by the bacteria's own metabolic processes of fermentation in response to the enzymes produced by autolysis, that naturally decompose the body, the byproducts of which then cause the physical changes in the corpse (Debruyn and Hauther, 2017; Adserias-Garriga et al. 2017; Javan et al. 2016). These studies show successional changes from microbes indigenous to the living body in the fresh stage of decay to the domination of bacterial groups indigenous to the gut microbiome that is spread by liquefication of tissue and purge. Then to a proliferation of obligate anaerobes in late decay as oxygen levels decrease. Finally, as the body is skeletonized and enters dry decay, a microbiome that is more similar to the native soil communities dominate (Damman et al.,2015, Debruyn and Hauther, 2017; Adserias-Garriga et al. 2017; Javan et al. 2016). Because these stages of decay have been timed out in previous research and we know the growth rates of some of these bacterial species, the addition of the research done by these scientists allows for the possible creation of a robust microbial clock that can be used to determine a PMI.

Biotic and Abiotic Factors that May Influence Succession

Uncontrollable variables in the environment such as weather and soil composition can make typical methods of estimating the PMI difficult. For example, the insects or maggots that are present on a corpse are quickly accessed for their stage of development, which can give a rough estimate for the time of death. However, the developmental growth rate of these insects can be drastically affected by temperature, weather, and geography. Insect activity is almost nonexistent in colder regions of the world. In contrast, hotter weather can greatly speed up insect development, making this method somewhat unreliable (Johnson et al., 2016). Although it has been typically thought that the thanatomicrobiome is independent of external abiotic and biotic factors (Javan et al. 2016), other research suggests that these factors may have a greater influence. Some research has shown that the ambient temperature could have an effect on the thanatomicrobiome and the rate of its development (Lawrence et al. 2019). Also because the necrobiome exists outside the body, it is directly influenced by external factors in the environment for which a body decays (Carter et al 2015). The following section will examine some of the environmental factors as well as the influences of ecological preferences and differences between males and females.

Temperature

In 2015, a study investigated the seasonal variations between the necrobiome in the summer and winter, focusing on the grave soil necrobiome for swine corpses (Sus scrofa *domesticus*). This research found that temperature had affected the decomposition rate of the carcasses (Carter et al., 2016). The swine were frozen in the winter season, which resulted in little visible decomposition until 30 days postmortem and in no

observed maggot activity until 25 days postmortem. In contrast, in the summer, insect activity was found to be ravenous and led to a rapid decay of the carcasses, and advanced decomposition was observed at just 9 days postmortem. Although carcasses in both seasons reached a similar state of decomposition by 60 days postmortem, the majority of decomposition in the winter only occurred after 15 days postmortem whereas most decomposition in the summer occurred prior to 15 days postmortem (Carter et al., 2016).

This study found that the most abundant bacterial phylum in all grave soils was Verrucomicrobia (Carter et al., 2016) Additionally, Solirubrobacterales (phylum Actinobacteria) and Psychrobacter *spp.* (P.roteobacteria: Gammaproteobacteria) proliferated much more in the winter, whereas Sphingobacterium *spp*. and Chitinophagaceae (Bacteroidetes: Sphingobacteriales) existed in much higher abundance in the summer season (Carter et al., 2016). Additionally, this research observed a greater presence of eukaryotes such as the protozoan Kinetoplastida (phylum Euglenozoa), nematode Rhabditidae, slime mold Fonticula alba, amoeba Euamoebida, fungus Eurotiomycetes, and fungus Tremellomycetes in the summer (Carter et al., 2016). This study suggests a few reasons for the development of necrobiome in this way. First, some of these eukaryotes use bacteria as food. Nematodes and amoebae, for example, are bacterivorous and their presence may be a response to an increase in their natural food supply. Second, the bacterial community changes the physical and chemical properties of the environment which allows the eukaryotes to proliferate. These changes include the creation of a higher level of moisture and alkalinity (8–9 pH) (Carter et al., 2016).

This study suggests that seasonality and the ecology of the location where decomposition began may be important factors to consider when determining a robust analysis of the microbiome for a PMI. For example, the presence of Psychrobacter *spp.* may act as an indicator of winter decomposition, whereas Chitinophagaceae, a family of bacteria that consume insects and fungi chitin, may indicate decomposition in the warmer months (Carter et al., 2016). These factors will be important for death investigations to consider since they may indicate that the climate and, henceforth, location that someone died in or if the PMI spans many months and through many seasons.

Differences in the Sexes

Interestingly, numerous studies have observed postmortem differences between the sexes. For example female cadavers have a high amount of Pseudomonas and Proteobacteria, with Pseudomonas appearing exclusively in females (Javan et al. 2016 Johnson et al. 2016), while male cadavers had a high presence of Streptococcus, and only male corpses host Gram-positive Rothia (Javan et al. 2016). Other research shows that males also seem to have a higher abundance of Firmicutes, Bacilli, Lactobacillales, and Rhizobiales (Johnson et al., 2016). Unfortunately, further research has yet to be done to determine why these differences in the sexes exist or how some of these bacteria were able to proliferate in the sexes separately. Further research has the potential to improve the accuracy of determining the sexual identity of unknown body parts, such as limbs, that have been separated from the rest of the body at the time of death or during decomposition.

Ecology

The groups of bacteria that make up the thanatomicrobiome are guaranteed access to their host's nutrients and organic matter after death as they, of course, live inside their host. As such, some bacteria have been shown to develop opportunistic lifestyles (Preiswerks et al., 2018; Javan et al. 2016; Bell et al. 2018). Some groups of microbes act opportunistically as the host approaches death, instead of acting as a decomposer or obligate host associate microbe (Preiswerks et al., 2018). In one study on Daphnia *magna*, a sit-and-wait (SAW) strategy was observed where decomposers may colonize inertly in a living host and wait until the host dies (Preiswerks et al.,2018). SAW strategy decomposers are guaranteed a chance of success, as every host will die eventually.

This study suggests that there may be an ecological relationship between the groups of microbes that develop in a host as that host approaches death and the microbes that decompose that host after death. Once the host dies, the SAW decomposers inside can begin their metabolic processes and break down the host's tissues. This in turn floods the surrounding environment with nutrients, allowing nearby microbes to feed and break down tissue from the outside. This suggests that SAW decomposers need a host in order to proliferate and that some microbes need the nutrients produced by the SAW decomposers to also proliferate. However, more research must be done as it is unknown how these microbes are able to develop within a still-living body and evade the immune system and what role they play as their host ages and begins to die.

The Universal Presence of Clostridium

Many of the members of the microbiota in late stages of decay are obligate anaerobes. In the early stages of decomposition, oxygen is what pushes the efficiency of organic matter to be broken down by aerobic bacteria like Staphylococcaceae (Javan et al. 2017). However, as oxygen is depleted, anaerobic bacteria like those in the Clostridiales order are able to dominate the body.

A majority of studies done on the microbes within the human body have shown that Clostridium *spp.* is a major contributor to the thanatomicrobiome and the necrobiome (Javan et al. 2016, Hyde et al. 2016, Johnson et al. 2016, Bell et al. 2018, Debruyn and Hauther, 2017; Adserias-Garriga et al. 2017; Carter et al. 2015). Nearly all studies found Firmicutes (a phylum of bacteria that includes Clostridium) to be stable biomarker across thanatomicrobiome communities within multiple zones of the body (Javan et al. 2016, Hyde et al. 2016, Johnson et al. 2016, Bell et al. 2018, Debruyn and Hauther, 2017; Adserias-Garriga et al. 2017; Carter et al. 2015). More recent developments found that Clostridium *spp.*, which typically were thought to dominate longer PMIs (10 days according to Javan et al. 2016), may also be the dominant species even at shorter time intervals of even just PMI = 4 hours. One study found in all cadaver samples that the highest percentage of bacteria observed were Clostridiales, and seven of the top species identified were Clostridium *spp.,* which was found in 95% of samples (Javan et al., 2017).

This information suggests that forensic scientists may only need to sequence out and search for Firmicutes rather than the entire microbiota of a corpse to determine PMI. Gram-positive, anaerobic extremophiles, clostridiales are a common order of bacteria

located in healthy intestines to aid in the digestion of our food. Clostridium *spp.* live within the mucosal layers of the intestines and they metabolize carbohydrates into acetic acid, acetone, butanoic acid, butanol, and ethanol which then can be used by other bacteria to ferment into pyruvate as a source of energy (Javan et al. 2017). With these characteristics, Clostridales may be a keystone species in the thanatomicrobiome, allowing other species to also proliferate.

Research suggests three factors contribute to the ubiquitous presence of Clostridium *spp.* First, Clostridium has an extremely fast population growth rate, with some species (C. *perfringens*) having a doubling time of 7.4 min at optimal temperatures (Javan et al., 2017). Second, Clostridium produces collagenases, which are normally used to digest collagen fibers in food. After the death of the host however, with the immune system no longer functioning, these bacteria can use their collagenase to pass through colon epithelial surfaces and mucosal layers and then migrate to other parts of the corpse to proliferate. Lastly, Clostridium is an obligate anaerobe and the lack of oxygenated blood in a corpse has been shown to allow the growth of many species of anaerobic bacteria without competition from aerobic bacteria. The presence of Clostridium may be the most important biomarker in the use of microbiota as a forensics tool because of its consistent growth rate and important role in the process of decomposition and overall microbial community growth.

Ultimately, the development of Clostridium throughout each stage of decomposition may be the central focus for research down the line. This group of species has shown to dominate in numbers at late stages of decomposition and its presence has also shown to affect the development of other groups of bacteria. Focusing on this group may make it easier for researchers in the future to create a microbial clock of decay.

Conclusion

Although much progress has been made researching the human thanatomicrobiome, many gaps still exist in the research. For example, no formal, robust catalog of these microbiota communities exists, at least not to the same degree of the Human Microbiome Project. Many of these studies have been done using culture-based techniques but unfortunately many bacterial taxonomic groups do not grow in laboratory cultures and thus create gaps in the research knowledge (Hyde et al. 2015). Furthermore, some of these research gaps include the presence of Pseudomonas in females exclusively and the role of microbiota in aging hosts. This research also has yet to be actually applied in death investigations by a medical examiner or forensic pathologist because the sequencing technique and classification are not yet efficient enough to be used in a timely manner and the data on all types of species in the thanatomicrobiome and necrobiome is insufficient. Therefore it has been deemed not ready to be used as evidence in such investigations or legal trials.

To address this lack of application, researchers may consider developing a machine type approach to analyzing data and predicting PMI; as well as improving the effectiveness of the traditional 16S rRNA Gene Amplicon sequencing used to identify bacteria. As this field is in its infancy there is still a long way to go, however, the

excitement among scientists shows a productive future ahead for this research. With the introduction of the Human Thanatomicrobiome Project by Gulnaz Javan of Alabama State University, a leading researcher on this subject, we may see a new way for forensic pathologists to handle their investigations.

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