Dental Calculus: Future in Forensics

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Dental calculus is a calcified biofilm that has sparked interest in recent years due to its rich molecular composition and its bone-like post-mortem preservation. Dental calculus is a natural and common part of the mouth. Because of this, it represents a potentially abundant resource for research. Dental calculus traps biomolecules present in the mouth during its formation, including food debris, bacteria, and host cells. As such, dental calculus holds promise for multiple fields of molecular research. Since dental calculus naturally occurs in humans, it could be an important substrate and valuable resource for use in forensic sciences.

What is dental calculus?

Dental calculus, also known as tartar, forms when plaque on the teeth becomes mineralized. Plaque refers to a soft bacterial film coating the teeth. This can be removed by maintaining a regular dental routine. However, when dental hygiene is not well maintained, the soft film will harden to form a mineralized matrix on the teeth referred to as dental calculus. This can no longer be removed by regular personal dental hygiene but requires scraping by a dentist.

Dental calculus has the same general makeup in all organisms, but the exact composition varies by individual. Many factors play a role in variation in composition between individuals. Charlier et al. (2010) provide a short list of factors, including food, salivary pH, oral hygiene, immune system strength, environment, oral anatomy, salivary flow, masticatory disorders, ethnic origin, age, and more. In summary, variation can be due to genetic factors, personal habits, and environment. However, it almost always remains true that dental calculus contains bacteria and other cells present in the saliva.

In the process of mineralization of dental calculus from plaque, calcium phosphates are involved in rapid intercellular and intracellular crystal formation. One of the calcium phosphates

involved is hydroxyapatite, which is of interest since it strongly binds DNA (Mann et al. 2018). Calcium cations especially help to stabilize and preserve DNA molecules (Preus et al. 2011). DNA holds a negative charge due to a phosphate group present in the backbone. The negative charges on the backbones of two strands of DNA usually repel each other. When calcium cations are present, this weakens the negative charge, causing the two DNA strands to more tightly bind to one another. This process leads to increased stability of whatever DNA molecules may be present in the saliva at the time of calcification. Brundin et al. (2013) found that DNA bound to hydroxyapatite lasted in different media (water, sera, and DNase I) for up to 3 months, while DNA without hydroxyapatite in the same media lasted only 3 weeks. The ability of ions in hydroxyapatite to stabilize DNA molecules leads to greater preservation.

Strands of host DNA – in this case, human DNA, when present in dental calculus – tends to be fragmented, perhaps due to how they are incorporated into the dental calculus matrix (Mann et al 2018). Human proteins in dental calculus consist mostly of neutrophilic innate immune system proteins released during infection and inflammation (Warriner et al 2014). Other potential sources of DNA in dental calculus include epithelial cell, blood, saliva, and gingival crevicular fluid (Black et al. 2011; Ozga et al. 2016; Ziesemer et al. 2019). The high rate of cell incorporation from immune responses and inflammation may correlate with the shorter length of host DNA in dental calculus (Ziesemer et al. 2019). Mann et al. (2018) suggest that DNA is actively incorporated through an immune response by neutrophils known as NETosis, which causes chromatin decondensation and destruction of the nuclear envelope, causing DNA in dental calculus to have a higher level of fragmentation.

Dental calculus is a vastly valuable resource; it can potentially preserve anything in a person's mouth, including food particles, bacteria, and host cells, within which proteins and

DNA are preserved. All these can be used in different fields and capacities to learn more about humans past and present.

Archaeological background of dental calculus research

Dental calculus research had its start in the investigation of archaeological mortuary samples. The characteristics of dental calculus, such as its high preservation and variety of molecules from inside and outside of the human body, make it a useful tool for archaeological investigation. Archaeological interest has propelled dental calculus research, leading to investigations that have important implications for modern humans and forensics.

The first instance of archaeological analysis of dental calculus can be found in a study by Armitage (1975), which investigated opal phytoliths in ungulates (large mammals with hooves) by extracting them from dental calculus, with the goal of learning more about diet. This investigation created a precedent for removal and analysis of dental calculus and is referenced in many papers following it.

Charlier et al. (2010) later researched how dental calculus could be used to identify the region of origin of archaeological remains based on bacteria, as well as how it could be used to reconstitute ailments and occupational habits. Additionally, they proposed that dental calculus could be used to infer manner of death. Charlier et al. (2010) investigate dental calculus through a lens of forensic anthropology using archaeological samples from multiple sites. Host DNA in dental calculus was not considered during the study. However, this was an early study that started asking questions about how dental calculus could be used in forensics, a theme that became more developed in later studies.

More recent studies have investigated microbial DNA in dental calculus. Weyrich et al. (2017) studied DNA preserved in dental calculus to learn more about behavior, diet, and disease

in Neanderthals. Macki et al. (2017) similarly used DNA in dental calculus to learn more about diet, disease, and health in Roman age humans. Both studies investigated bacterial DNA and microfossils in the dental calculus and were able to make significant estimates about diet, health, and culture of the groups they were studying. Macki et al. (2017) claimed that dental calculus is a reliable source of DNA, with a minimal sample size required for analysis.

These and other studies desired to learn more about the evolution of the human oral microbiome and its implications in diet and disease in both ancient and modern humans (Weyrich et al 2017; Warriner et al 2015). These types of studies are important as comparative studies for modern oral health, as well as in investigating the 'microbial fingerprint' (Weyrich et al 2015), the microbial signature of an individual. Additionally, Macki et al. (2017) were interested in adding this information to the 'osteobiography', which includes characteristics of the skeleton based on evidence of disease and bacteria.

These archaeological investigations have significantly contributed to DNA research in general. In an earlier study of DNA in dental calculus, Preus et al. (2011) demonstrated that "fairly long" sequences of ancient DNA (aDNA) could be identified in bacteria preserved in dental calculus of "considerable age". Following this, many studies investigated the implications of isolating aDNA from dental calculus and the importance of this matrix being able to preserve nucleic acids.

Additionally, multiple studies have found that dental calculus is important for archaeology because it is ubiquitous in ancient populations and preserves aDNA well (Preus et al. 2011; Warriner et al. 2015). Although dental calculus was more commonly present in ancient populations due to a lack of dentistry and daily brushing (Weyrich et al. 2015; Macki et al. 2017)

the characteristics that make dental calculus a good source of well-preserved DNA also hold true for more modern samples.

The human oral microbiome

The human oral microbiome is a prevalent area of current research in the field of dental calculus. Studies have referenced its importance for human health, as well as its potential importance in forensics. Research into the oral microbiome has also led to advancements that will aid in DNA research.

In an earlier study into the microbiome of the mouth, Xie et al. (2010) investigated the molecular makeup of dental plaque (the precursor of dental calculus). A metagenomic analysis of healthy human plaque from one individual showed DNA of harmful pathogens, but also genes that encode for proteins that are involved in resistance to toxic compounds. This shows that the mouth has its own defense system to its microbial community. Xie et al. (2010) also found that one third of the sequences within the plaque were of host origin. This study only looked at the precursor to dental calculus and a very small sample size that could be expanded upon in the future. However, this study into the microbiome still lends important information about the potential genetic makeup of dental calculus. If one third of the genes in plaque is of host origin, this is promising for future studies into host DNA in dental calculus.

In another study, Warriner et al. (2014) built a next-gen shotgun sequencing library of biomolecules in the human mouth, done using only milligrams of dental calculus. Using this, Warriner et al. (2014) explored the taxonomy of species in dental calculus and attempted protein functional characterization within the microbiome. The study also compared ancient samples and modern samples with known dental history, which opens the door to comparative studies of ancient and modern dental calculus. Warriner et al. (2014) found that one third of proteins in

ancient dental calculus are shared with modern humans, along with highly similar functionality. In addition, most proteins were related to the host immune system. This helps in identifying the origin and function of proteins in dental calculus, which could lead to conclusions about the abundance and quality of host DNA present. While this study did not focus on host DNA, it is important in the stream of research for further characterizing the microbiome. It also shows the potential for further study of dental calculus in modern humans.

Focusing on obtaining bacterial DNA for archaeological studies, De La Fuente et al. (2013) developed a methodology for isolating and analyzing DNA from bacteria in dental calculus. The study focused on five bacterial types (*Antinomyces naeslundii*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Streptococcus gordonii*, and *Streptococcus mutans)*. In addition, De La Fuentes at al. (2013) argued that host DNA should be able to be amplified, successfully amplifying hypervariable region 1 of human mtDNA. This study demonstrated the ability to isolate mtDNA, which has important implications for forensic DNA studies.

Multiple microbial studies have aided in learning more about the characteristics of dental calculus. Brundin et al. (2013) gave a reason behind the preservation of DNA in dental calculus, finding that mineral growth around and within oral bacterial cells may directly aid in nucleic acid survival. Mann et al. (2018) found that DNA in dental calculus is significantly abundant, even more so that in dentin; however, it is mostly comprised of bacterial DNA. Mann et al. (2018) also found that in heavily decayed remains, the majority of human DNA in dental calculus was human-associated oral taxa, while in dentin it is mostly environmental oral taxa. This shows that dental calculus has much less contamination than dentin, making it a more reliable source of study. Both studies focus on the microbial environment of dental calculus but show that

molecules within dental calculus are highly preserved, solidifying dental calculus as an important resource for study.

Host DNA

Multiple studies have attempted to isolate endogenous human nucleic acids from dental calculus with different proposed applications. The most successful so far has been the isolation and analysis of mitochondrial DNA, but isolation of RNA and of nuclear DNA has also been attempted with some success.

Mitochondrial DNA

Multiple studies show that mitochondrial DNA (mtDNA) can be isolated from dental calculus in quantities significant enough for analysis. The earliest study with successful isolation and analysis of host mtDNA from dental calculus is that of Black et al. (2011), who successfully isolated and analyzed host mtDNA from remains of Native Americans. In addition, they successfully assigned them to Haplogroup D with geographic origins in northern California (Black et al 2011). This study not only showed the efficacy of isolating mtDNA from dental calculus, but it also proposed and applied potential forensic methods for identification of remains using that mtDNA. In addition, this study also went into more information about the characteristics of dental calculus. Black et al. (2011) suggest that dental calculus contains three common host DNA sources: epithelial cells, saliva, and proteins. These three sources could be important for future research and DNA isolation. Black et al suggest future research with larger samples sizes, blind testing of known mtDNA haplogroups, and samples of varying ages.

A subsequent study by Ozga et al. (2016) also attempted to isolate host mtDNA from dental calculus of archaeological samples, three out of six of which had previously tested negative for DNA preservation in bone. From this study, Ozga et al. concluded that

mitochondrial DNA in dental calculus is of substantial quality and high quantity, making it an excellent source for study and for mitogenome reconstruction. They highlight the ability of dental calculus to be used for ancestry reconstruction, especially when destruction of remains is not viable.

Ozga et al. (2016) also talk about potential sources of DNA in dental calculus. The three main proteins found in the dental calculus included follicular dendritic cell-secreted protein, alpha amylase I, and hemoglobin, probably coming from gingival crevicular fluid, saliva, and blood.

A recent study by Modi et al. (2020) tested methods using the same sample of dental calculus to get archaeobotanical information and to isolate mtDNA They successfully procured human mitochondrial DNA in two out of three samples. This study successfully showed the resource-saving potential of multiple tests on the sample dental calculus sample, as well as the high efficacy of mitochondrial DNA isolation.

miRNA

Lippolis et al. (2018) successfully isolated and quantified microRNA (miRNA) from ancient and modern samples using real time (RT)-PCR. Lippolis et al. conclude that miRNA can survive long-term post-mortem. In addition, Lippolis et al. (2018) claim that analyzing saliva-derived miRNAs in dental calculus could provide valuable information to identify individuals post-mortem, especially in mass calamities, fires, or explosions, after which teeth are often the only tissue available for analysis. This study is a promising proposition, but also contains areas for improvement. Archaeological samples were collected from graves and modern samples were collected from living humans in a dental clinic (Lippolis et al. 2018). Neither of these sources properly represent the level of degradation common in forensic cases. Future studies could

attempt to replicate the work of Lippolis et al. with more degraded and a wider variety of samples.

Whole human genome

In a recent study, Ziesemer et al. (2019) attempted to isolate human genomic material from dental calculus and were able to successfully enrich human endogenous content fourfold. This shows that human nuclear DNA is also able to be successfully isolated from dental calculus. As with other studies, Ziesemer et al. (2019) suggest that DNA sources were saliva and gingival crevicular fluid. They sought a genome-wide sequence since it can be used to estimate sex, ancestry, kinship, and genetic relations, and provide information on human-environment interactions. Isolation of human genomic material in significant amounts would have incredible implications for the use of dental calculus in forensics. In addition, Ziesemer et al. (2019) found that the quality of general DNA in dental calculus is higher than that in dentin, another common source of DNA. The DNA appears to be less damaged due to the preservation processes inherent in dental calculus formation. However, the researchers acknowledge that the yield in enrichment of mitochondrial DNA is significantly higher than nuclear DNA and suggest further improvement of whole genome enrichment techniques before applying their methods more widely.

Host DNA in Dental Calculus vs. Dentin

Dentin, the layer of teeth between the enamel and the pulp, has been used in archaeology and forensics as a means of retrieving DNA from teeth. However, removing dentin is destructive to the tooth since the enamel needs to be removed and the dentin pulverized. Multiple studies have compared the DNA content and its usefulness in dentin and dental calculus.

Dentin, on average, has a higher percentage of human DNA than dental calculus since it is originating from a source made up entirely of human tissue. However, though dentin has a relatively small amount of host DNA, the amount you find in dental calculus is more consistent across samples than dentin, which varies widely.

The percent of host DNA making up dental calculus, according to Mann et al. (2018), has a mean of 0.08% +/- 0.08%, with a range of 0.007 to 0.47%. Ziesemer et al. (2019) estimated the average quantity of human DNA in dental calculus to be 0.5-210 pg/mg. This is comparatively less DNA than other sources of host DNA in remains. However, removal and analysis of dental calculus is nondestructive, unlike other methods that yield more DNA. While the amount of DNA in dental calculus is not high enough to perform procedures that require higher quantities, like shotgun sequencing, it is still a valuable source to recover DNA using DNA capture methods.

Dentin, while generally higher in endogenous human DNA content, is at higher risk of contamination post-mortem. According to Warriner et al. (2015), the most common place or teeth to experience taphonomic changes is the pulp and the dentin immediately surrounding the pulp, especially near the apical foramen, which is connected to the rest of the body by nerves and blood vessels. This is an entry point for environmental microbes. This leads to the conclusion that DNA from dentin is more likely to have environmental contamination form post-mortem processes compared to dental calculus, which like bone, is mineralized and forms a matrix almost impenetrable by outside organisms and molecules. Warriner et al. (2015) claims that this complicates studies saying that dental pulp, cementum, and the root tip should be used preferentially to recover host DNA. On the contrary, these sites appear to be altered post-mortem due to environmental exposure.

Mann et al. (2018) found that dental calculus has a higher DNA content in archaeological samples compared with dentin, which they say reflects biological differences between the two, including cellular composition, structure during life, and decomposition patterns after death. Therefore, DNA in dental calculus is especially useful in archaeological samples.

When comparing dental calculus and dentin, it is clear that dental calculus is a more consistent and less contaminated source of host DNA in both archaeological and modern samples than dentin.

Methods for retrieval and analysis of host DNA from dental calculus

Methods for isolating host DNA from dental calculus are important to examine to learn more about what works, what doesn't work, and what should be applied in the future. All studies mentioned have slightly different methodologies based on what specifically is being targeted and what the goal of the research is.

miRNA

Lippolis et al. (2018)

As the only study specifically targeting RNA, this method is the most variable from the rest. Samples are subjected to UV irradiation to get rid of surface contamination, then submerged in a solution of 4% bleach and rinsed in 90% ethanol for 3 minutes to get rid of the bleach. The dental calculus is then pulverized in liquid nitrogen with a mortar and pestle, and UV irradiated overnight.

RNA is extracted using a phenol-chloroform method, followed by purification using a Qiagen miRNeasy kit. Total RNA (including bacterial) was quantified using a spectrophotometer (A260/280) and a Qubit Fluorometer. Bacterial RNA was then isolated from human RNA using

a MICROBEnrich kit (Thermo Fisher Scientific). miRNA levels were then evaluated by RT-PCR.

Mitochondrial DNA

Black et al. (2011)

Black et al.'s study focused on isolating DNA from dental calculus in a way they believed would be appropriate for Native American remains, ensured that approximately half of the total calculus per individual was reserved for future analysis. mtDNA of individuals working on the samples were analyzed to rule them out as sources of DNA contamination.

Dental calculus was processed using an extraction procedure common for bone because of its density. The Geneclean Kit for Ancient DNA protocol was used. The sample was pre-incubated in Proteinase-K to digest contaminating proteins and to protect DNA by degrading nucleases. The pre-incubation was extended from the recommended 12-15 hours to 70 hours.

10 microliters of extraction product from each sample was amplified using QIAGEN REPLI-g for FFPE (formalin-fixed and paraffin-embedded tissues). Extraction products and FFPE products were amplified using specific primers for the four American Indian Haplogroups A, B, C, and D. Qiagen Fast Cycling PCR Kit was used for all PCR amplifications.

A restriction digest was performed to determine the presence or absence of restriction sites that identify mtDNA Haplogroups A, C, and D. Gel electrophoresis was then performed with samples that amplified Haplogroup B to determine the presence or absence of 9 bp deletion.

Ozga et al. (2016)

Ozga et al.'s goal was whole mitogenome reconstruction. Dental calculus was extracted from archaeological samples in a dedicated ancient DNA facility at the University of Oklahoma Laboratories (LMAMR). Prior to extraction, dental calculus samples were UV-irradiated for 1

minute on each side. To further remove any contaminants, the samples were agitated in 0.5 M EDTA solution for 15 minutes. EDTA is a chelating agent that sequesters metal ions that are needed for DNAse activity, thereby preventing DNase from breaking down DNA. The samples were then resuspended in 1 mL of 0.5 M EDTA solution and incubated overnight at room temperature. 100 microliters of proteinase K solution were added at 37 °C for 8 hours followed by further digestion under agitation at room temperature until the dental calculus was decalcified.

DNA was extracted using phenol-chloroform separation. Extracted DNA was then purified by silica adsorption and eluted into 30 microliters of EB buffer. 1 microliter of each extract was quantified using Qubit.

In modern samples, mtDNA was extracted in the same way, but without the decontamination steps. In addition, isolated DNA was sheared by sonication to a target length of 200 bp to reach the typical length of more degraded samples.

A Shotgun Illumina library was constructed for the ancient DNA samples. 100 ng of DNA was constructed into indexed libraries using NEBNext DNA Library Prep Master for 454, but the SPRI bead purification step was replaced with silica adsorption. Modern samples were prepared using KAPA HiFi Uracil+ protocol with 8 amplification cycles.

Samples were run through two Illumina MiSeq runs at the DNASU sequencing core at Arizona State University. Two samples were also selected for shotgun sequencing to determine mtDNA content, which were then sequenced with Illumina Hi-Seq at the Yale Center for genomic analysis.

Modi et al. (2020)

The aim of Modi et al.'s study was to compare multiple common methods to extract DNA and archaeobotanical information to get the highest possible yield of both. DNA was

extracted in the clean-room facilities of the Laboratory of Molecular Anthropology and Paleogenetics, University of Florence, using a silica-based protocol specifically designed by Dabney et al. (2013) to improve the recovery of short, degraded molecules. 5 different methods of decalcification and protein digestion were used. The first two samples were incubated in either 1% sodium hypochlorite (bleach) or in a 0.5 M solution of EDTA. The last three methods combined different amounts of reagents under varying conditions.

Two different extraction methods were used aiming to isolate DNA. In the first procedure, samples were washed with 1 mL EDTA to remove environmental contaminants. The dental calculus was then crushed to obtain a coarse powder, then incubated in 1 mL extraction buffer (0.45 M EDTA, 0.25 mg/mL Proteinase K, 0.05% Tween 20). After incubation, DNA was extracted from the supernatant using a silica-based protocol.

In the second procedure, samples were rinsed shortly with boiling water, then incubated in a 10% solution of hexametaphosphate for 24 hours. The sample was then ultra-sonicated and incubated in 10% HCl for 12 hours. After sonication, the samples were washed twice with H2O then incubated in the same extraction buffer and incubation conditions as the first procedure. It was found that this procedure resulted in a major decrease in DNA retention (Modi et al 2020).

DNA was quantified using a Qubit Fluorometer. 10 cycles of PCR were performed. DNA was sequenced using an Illumina MiSeq.

Nuclear DNA

Ziesemer et al. (2019)

Ziesemer et al aimed to isolate the human genome from dental calculus using whole human genome capture. In the tooth samples used, the tooth surface was first washed with 2% sodium hypochlorite (bleach), followed by molecular biology grade water. Calculus was removed from

the tooth surface using a scaler. The dental calculus was further decontaminated by UV irradiation in crosslinker for 1 minute on each side. After this, the calculus was washed in 1 mL of 0.5 M EDTA under centrifuge rotation for 15 minutes. The pellet formed was then digested in 0.45 M EDTA and 10% Proteinase K while heating 37 degrees C to 55 degrees C for 8-12 hours.

DNA was extracted from the DNA-containing supernatant using phenol-chloroform solution. The sample was further purified and concentrated using a QIAGEN MinElute silica spin column. DNA was quantified using a Qubit Fluorometer 2.0 High Sensitivity Assay. An Illumina library was built, and the human genome was enriched using MYBait human whole genome enrichment. Illumina Hi-Seq was performed on pre-enrichment and post-enrichment samples.

Methods: Conclusions

The consensus methods for multiple studies to isolate DNA from dental calculus tell what techniques are successful, and what should be replicated in the future. For every study, using methods to limit and account for contamination were important. Most of the studies used UV irradiation to decontaminate, and most used some form of bleach. Every study that isolated DNA used Proteinase K to decontaminate in some capacity, and most used 0.5 M EDTA to chelate. Additionally, it was important in each study that a clean lab was used.

Three of five studies above extracted DNA using a phenol-chloroform extraction method. In these cases, extraction was followed by silica-based purification. In four of five cases, A Qubit Fluorometer was used for quantification. Ziesemer et al. (2019) specifically used a high sensitivity assay to quantify DNA since it was extracted in such low quantities.

Multiple studies using Illumina next-generation sequencing to sequence DNA due to the large size of the sequences. Ozga et al. (2016) and Modi et al. (2020) both used Illumina MiSeq for the mitochondrial genome, while Ziesemer et al. (2019) used Illumina Hi-Seq since they were trying to sequence human genomic content.

From this, we know that it is of utmost importance that samples are decontaminated and prepared in a manner normal for DNA analysis. The Phenol-chloroform extraction method seems to be common and successful. Enrichment and sequencing are dependent on the type of study and what needs to be sequenced.

Forensic DNA

Current forensic techniques are continually developing to be able to obtain information from samples that are highly degraded or contaminated by their environment. In dental calculus, where DNA exists with little contamination but in small amounts and short sequences, it is very possible that forensic techniques could already be used to obtain information from DNA in dental calculus or will be able to in the near future.

The standard technique used in most forensic DNA typing laboratories is isolating autosomal genetic markers called STR (short tandem repeat) loci. STRs are repetitive sequences in the DNA that are highly prone to mutation, and therefore can often be used to individualize a sample when compared to DNA of a known individual or someone related to them. According to Alvarez-Cubero et al. (2012), a standard of 10-17 STR loci are sufficient to estimate the identity of an individual. There are, however, some cases in which STR loci are not sufficient, such as in cases where there is a mixture of a large amount of female DNA and some male DNA or when a relative is several generations from the individual.

In these cases, additional testing of mtDNA helps with individualization. mtDNA is especially useful for highly degraded samples or samples of low quality. mtDNA, present in the mitochondria of a cell, has hundreds of thousands of copies compared to nuclear DNA that only has two copies per cell. mtDNA testing is therefore especially useful for dental calculus, in which DNA is present in low quantities, and often degraded from processes during life.

A newer approach being explored in forensic DNA analysis is using SNPs (single nucleotide polymorphisms) to individualize. As their name suggests, SNPs only occur at the site of a single nucleotide. Because of this, primers for shorter sequences, and therefore shorter amplicons are needed to capture SNPs. Because of this, samples with degraded sequences or shorter sequences, like those common in dental calculus, can be typed with power equivalent to STRs when many SNPs are present. While SNPs are not currently widely in use, they are a promising future direction, especially in getting forensic information from sources with degraded DNA like dental calculus.

Benefits of the use of dental calculus in forensics

The unique characteristics of dental calculus make it especially useful for forensics. This mostly stems from the way that dental calculus is formed in the mouth. Dental calculus entraps biomolecules in a calcified matrix. If trying to perform microbial analysis, dental calculus is an excellent substrate and, according to Macki et al. (2017), less than 50 mg is required for analysis. Dental calculus forms incrementally over time and highly preserves anything from plaque, trapping biomolecules and other debris that show a snapshot of the mouth from when it is formed. The result of the way that dental calculus is formed is that it becomes a “dense crystalline structure” that is “resistant to microbial attack, enzymatic action, and non-acidic

chemical alterations" (Mann et al 2018). Dental calculus, because of its dense calcified structure, is much less prone to environmental contamination than other DNA sources. In addition to its chemical properties, dental calculus is also physically difficult to penetrate, lending more to its resistance to outside contamination.

Dental calculus calcifies similarly to bone during life. Like bone, dental calculus does not undergo the same decomposition as other parts of the body. When all soft tissue that could possibly be used for forensic analysis is gone, dental calculus remains attached to the teeth. Additionally, if dental calculus is found on one tooth, it is more than likely on other teeth: this makes it easier to sample a small amount of dental calculus while leaving more for future sampling.

In fully skeletonized remains, skeletal tissue can still be used to find DNA. However, this is often destructive and results in both damage to the skeleton and loss of material. However, removing and analyzing dental calculus is considered nondestructive since it is not a host tissue. It is considered an "ectopic growth" (Macki et al. 2017) and a "calcified microbial biofilm" (Ozga et al. 2016), and therefore can be removed without causing any damage to the body itself. Dental calculus does not originate from the body and serves the body no purpose; instead, it is caused by the introduction of bacteria into the mouth, and the process of calcification happens to trap human biomolecules along with foreign material. When presented with teeth, a nondestructive analysis technique is always preferred. Because teeth possess identification value beyond that of DNA analysis alone, the tooth should not be arbitrarily destroyed. This can be avoided by using dental calculus.

Besides its inherent properties, dental calculus also has the advantage of the protection that teeth have in general. Teeth are protected by the jaw and the muscles of the mouth. Dental tissue

is a good DNA source because of its resistance to physical and chemical effects, as well as "environmental assaults such as incineration, immersion, trauma, mutilation and decomposition" (Lippolis et al. 2018). Using DNA from teeth provides a basis for reassociation of body parts that might not be otherwise possible because of decomposition.

In their study isolating mtDNA from dental calculus, Black et al. (2017) bring up benefits of dental calculus when it comes to analyzing and repatriating remains of Native Americans. Dental calculus is used as a source of DNA that can be analyzed nondestructively, as previously described. This is important for respecting cultural norms about treatment of remains that go against destructive analysis. Because removing dental calculus is less destructive than traditional methods of DNA analysis and dental calculus is not considered a human tissue, Black et al. (2011) believe that some tribes may be encouraged to allow DNA analysis. Black et al. (2011) emphasize the benefit that dental calculus can be used for indirect DNA analysis but can be directly linked to an individual.

While not mentioned in previous research to my knowledge, another potential benefit of dental calculus is the potential to identify remains of fully skeletonized adolescents in a non-destructive way. Since adolescent skeletons are not fully developed, using landmarks common in adults to estimate information about the individual – especially sex – are not useful. Dental calculus, which occurs in children, is a potential avenue by which adolescent skeletons can be identified non-destructively.

In addition, dental calculus is nearly ubiquitous in archaeological skeletal collections and ubiquitous in modern adults without good oral hygiene practices. Dental calculus is an abundant and rich resource that has not previously been regarded or used to its full capacity.

Downsides of dental calculus

There are still parts of using dental calculus that need to be improved upon, or that present caveats to the usefulness of dental calculus. One problem could be that not all communities would be willing to allow removal and analysis of dental calculus. Using dental calculus is considered to be nondestructive to human tissue and could provide an avenue to identify individuals that wouldn't be possible otherwise. However, some communities may not view it as non-destructive. These wishes need to be fully respected. There are also cases in which remains may be too compromised or degraded to remove any dental calculus.

Additionally, for modern humans from first world countries and higher income areas, dental calculus may not be as common because of higher upkeep of personal dental hygiene and accessibility to dental healthcare. It is possible that dental calculus would either not be present or be present in very small amounts in these areas. However, dental calculus is still a natural phenomenon that takes place in every individual, and if an individual is between dental visits or lacking good dental hygiene, dental calculus remains ubiquitous.

Future directions

Dental calculus is a new and emerging field, and as such, there are many future directions that can and should be pursued. Mitochondrial DNA in dental calculus has been shown to be of high quantity and has been isolated and analyzed successfully several times. mtDNA in forensics is, in itself, still being explored for its efficacy in forensic sciences. Hopefully, the two will in tandem continue to be researched and developed. While we are sometime away from this, further refinement of isolation of DNA from dental calculus will be needed in order to pass forensic standards. This is an important direction to go in, especially in cases where destruction of remains is limited or inhibited.

Another potential area of future research is improving upon whole genome enrichment of dental calculus samples. For the purposes of forensics, studying the efficacy of whole genome enrichment on modern samples could be beneficial. Sequence capture is especially helpful since, as pointed out by Ziesemer et al. (2019), it reduces the amount of material required for analysis and decreases experiment workload and cost.

Even if whole genome capture cannot be performed with high efficacy, isolation and analysis of STRs and SNPs should be attempted. Since DNA in dental calculus is often shorter in sequence due to the processes by which it is formed, using STRs to learn more about an individual could be more useful than techniques like whole genome capture. SNPs would be especially helpful in this since an even shorter sequence needs to be isolated. SNPs are also still developing in forensics, but further tandem development of the use of STRs and DNA in dental calculus is promising.

Overall, dental calculus research is a new field with many directions of future research that it could be useful. Dental calculus could store information about diet, environment, disease, the evolution of the microbiome, and the genome. Further research into how the many uses of dental calculus can be exploited would lend to the development of a rich and powerful resource in forensics.

Conclusion

The unique characteristics and preservation of dental calculus has made it a research topic of interest in recent years. The two most prominent current fields of dental calculus research are in archaeology and the human oral microbiome. Dental calculus is especially useful in archaeology because of its long-term preservation and resistance to contamination. Biomolecules including bacteria, food particles, and host cells become trapped in dental calculus

when it is formed. These same characteristics that make dental calculus useful in archaeology and the study of the microbiome make it useful for molecular research of modern samples. These two prominent fields of research have helped to reveal important characteristics of dental calculus and its potential usefulness.

Isolation of host DNA has been attempted successfully multiple times using varying nucleic acids, showing that dental calculus is a viable source of host DNA. Methods from these studies show us what has been successful in isolating host DNA and could be applied to future techniques, including forensics. In addition, modern forensic DNA analysis techniques could be applied to dental calculus.

Dental calculus is a resource with emerging importance that should be explored. It could be especially useful in forensic cases where destructive analysis is not permitted, or where it is not easy to get information using only forensic anthropology techniques as in the case of determining the sex of an adolescent. Future research should include improving upon enrichment of host nuclear DNA and attempting modern forensic techniques like STR and SNP analysis.

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