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DIGGING DEEPER: ENHANCING ARCHAEOPARASITOLOGY BY COMBINING MOLECULAR METHODS WITH TRADITIONAL MORPHOLOGICAL APPROACHES

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A DISSERTATION APPROVED FOR THE
DEPARTMENT OF ANTHROPOLOGY

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I dedicate this dissertation to the memory of my husband, Peter Riley Cleeland (1966-2012).

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Abstract

Coprolite science, human parasitism and ancient DNA methodology, converge most appropriately in the sub-specialty of archaeoparasitology – the study of prehistoric parasitism. In this study, we have applied targeted PCR to an archaeological sample from *La Cueva de los Muertos Chiquitos*, El Zape, Durango, Mexico, ~ AD 600. The addition of molecular analysis, resulted in the identification of a rare human parasite, previously unidentified, and a clarification of ambiguous morphological parasite remains. Discovery of an unexpected parasite has implications for the interpretation of human health in this prehistoric site. An additional analysis of previously generated Whole Genome Shotgun (WGS) Next-Generation Sequencing (NGS) data, resulted in the development of a series of filters to increase certainty of taxonomic identifications. The results of the NGS data manipulation failed to identify parasites in the dataset, but provides a foundation for a discussion of future research and current deficiencies in the reference databases. It is recommended that a combined morphological and molecular approach is the most robust methodology for archaeoparasitological research. It is also recommended that resources be routed into the development of NGS targeted approaches. A final recommendation that increased systematic effort be applied to adding parasite reference sequences to publicly available reference databases. Given that human parasitism impacts one third of the world population, any technology that increases the information retrieved, while creating a cost effective and robust methodology, will benefit both modern

clinical researchers and prehistoric researchers. Therefore, work from prehistoric studies is directly applicable to the issue of parasitism in the modern world.

Chapter 1: Introduction

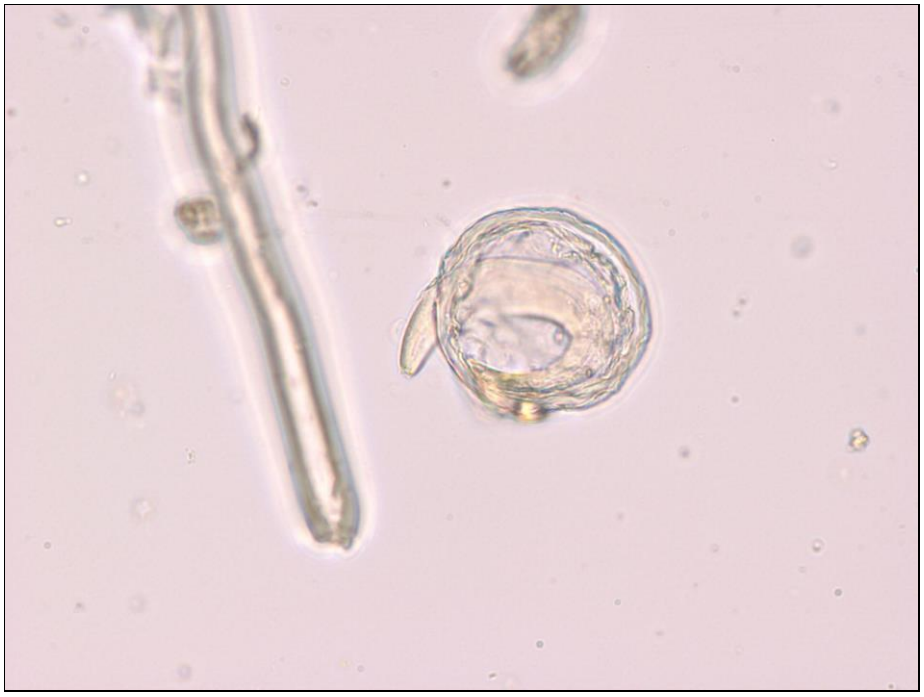

Figure 1: 1400 year-old Ascarid suspect from *La Cueva de los Muertos Chiquitos*, sample number 29, recovered from unit B4 during 1957 Excavations. Photo Taken at 400x magnification.

The very nature of science is discoveries, and the best of those discoveries are the ones you don't expect. ~ Neil deGrasse Tyson

When originally conceived, this dissertation intended to explore questions related to the links between diet and parasitism in prehistoric populations, using molecular methods to elucidate dietary components and parasite burdens among both hunting and gathering groups and agricultural groups, as differences in parasite loads have been noted in both prehistoric and modern populations (Reinhard, et al. 1985).The original hypotheses suggested that a more varied diet, would be found among hunter-gatherers. This hunter gatherer diet would include natural anti-helminthic plants, such as

Chenopodium or Black Walnut, which would have resulted in lower or non-existent parasite burden (Merckle 2010; Reinhard, et al. 1985; Reinhard, et al. 1987). On the other hand, agriculturalists were expected to have a less varied diet that resulted in the loss of anti-helminthic plants in the diet and thus, a higher parasite load. Unfortunately, it was not possible to obtain suitable and comparable samples of coprolites to explore this question adequately.

Two considerations shaped the ultimate objective of this study. First, through discussions with archaeological colleagues a reluctance to employ ancient DNA (aDNA) methods became evident. This reluctance was based on perceptions of the difficulty of the science to produce usable results, based largely on issues encountered in the earliest studies. Issues of concern included failure to obtain genetic information, potential to fail to obtain authentic aDNA, cost prohibitive analyses, and an inability to provide additional or otherwise unattainable information. All of these issues are of prime importance to archaeologists, when determining the most appropriate analyses for irreplaceable samples.

Second, in the thirty years since the first aDNA studies, many of the above issues have been addressed and resolved. However, applying aDNA technology to archaeoparasitological work is young, little more than a decade old and relatively few archaeoparasitological studies have involved the use of aDNA methods. Therefore the objectives of this study changed to determining whether or not using molecular technology to examine prehistoric parasitism

was both possible and uniquely informative. A number of assumptions are made in this study. These assumptions are:

1. Archaeologists have been reluctant to employ ancient DNA protocols in their studies, based on misconceptions and an information disconnect between molecular researchers and archaeologists (see Appendix C).
2. Parasite DNA is preserved in desiccated fecal samples, even in the absence of identifiable physical remains.
3. This DNA will be degraded and fragmented.
4. This DNA will be recoverable, as other degraded DNA is recoverable, using the techniques developed by ancient DNA research.
5. There are sufficient foundational reference materials available for taxonomic identification.

Based upon the above assumptions, the following questions were investigated in relation to molecular methods in examining prehistoric parasitism.

1. Can Ancient DNA methodology capture authentic genetic information for an organism in the absence of physical remains?
2. Are genetic reference databases sufficient for taxonomic identification to the genus and species level for any parasites encountered?
3. Will both types of molecular approaches Polymerase Chain Reaction (PCR) and Next Generation Sequencing (NGS) produce genetic information on the parasites included in the coprolite?

4. Will Whole Genome Shotgun sequencing data capture parasite specific genetic information and allow subsequent taxonomic identification?
5. Can information obtained through genetic analysis of coprolite remains enhance traditional morphological archaeoparasitology methods?
6. Can Ancient DNA (aDNA) analysis replace traditional microscopic morphological analysis?

The purpose of this dissertation is to demonstrate the methods necessary and procedures involved in the application of molecular analyses to archaeoparasitology. The study used two different approaches and methods of molecular analysis to answer the questions posited above about prehistoric parasitism. This study used two 1400 year old, putative human coprolites, labelled as Zape 23 and Zape 29. The coprolites were recovered from Unit B4 at the *La Cueva de los Muertos Chiquitos* archaeological site, in Zape, Durango, Mexico in 1957 (Brooks, et al. 1962). Zape 29 was eventually discarded due to weak and inconsistent results. Zape 23, however, routinely provided consistent, accurate and robust results.

Chapter Two will provide background information pertinent to this dissertation. It will first discuss the site and cultural history of the *La Cueva de los Muertos Chiquitos* site. It will then discuss a short history of coprolites, which are fossilized or desiccated fecal material. Coprolites are literally a treasure trove of information, but their importance was only fully recognized

rather recently. The information coprolites provide are of particular interest to archaeologists, bioarchaeologists, and paleoecologists.

Human parasitism will also be discussed in Chapter Two. Human parasitism is defined as the condition of a human being used as a host for a parasitic organism, which makes its home and sustains its life by co-opting the resources of the human body (Bogitsh, et al. 2013; Combes 2001). This is an extremely large topic and can cover ecto-parasites, which attach to the outside of the body, such as, ticks or lice. It can cover parasites, which invade the skin or other organs of the body, such as the trematodes. It can also refer to protozoan parasites that cause diseases such as dysentery, these include organisms such as *Giardia* or *Entomoeba*. Macroparasitism also applies to human health and social construction. Macroparasitism is the use of a host by large bodied parasites, sometimes associated with predator-prey relationships it can also be associated with social inequality and human on human parasitism. This can result in increased health disparities, including increased microparasitism between elite and commoner social groups. For this study, however, the topic is limited solely to the intestinal helminthes – endo-parasitic worms, which inhabit the human gastrointestinal system.

Ancient DNA will also be discussed in Chapter Two. A short history of its progression will be provided and a discussion of both Polymerase Chain Reaction (PCR) and Next-Generation Sequencing (NGS) platforms and methodological approaches will be defined.

The final set of information provided in the background of Chapter Two, is a caveat regarding the identification of the origin of the coprolite. Close living association between humans and canines can result in difficulty in species of origin identification for coprolite specimens. Despite a number of methodologies to rectify this difficulty, it remains a serious concern for the interpretation of coprolite findings (Bryant and Dean 2006).

Chapter Three covers all the methodologies used in this study. These include preparatory methods, such as, the development of positive control samples and the design and testing of primer sets, used to book-end and recover specific genetic sequences. The methods for PCR amplification, begin with sample preparation which includes sample dissection and rehydration, followed by DNA extraction and PCR amplification. Additional methodologies include sequencing, trimming and identifying sequence data, as well as phylogenetic tree building.

The use of previously generated data for the Zape 23 samples are used for the NGS methodology, no new data was generated for this section. As such, it provides a number of filtering processes and a series of reference based comparisons in the process of assigning identification to the sequence data.

Chapter Four presents the results and discussion of the various analyses. The determination of the coprolite's origin. The results of the targeted PCR analysis, as well as the results of the data mining of the previously generated NGS data set.

Chapter Five presents the conclusion to this study. A set of recommendations for future research and a final recommendation of the most appropriate use of molecular technologies in relation to archaeoparasitological research.

As noted in the assumptions above, there is a persistent reluctance on the part of archaeologists to undertake molecular analyses in relation to their work, even though the state of the science has become quite routine in many respects. Included in the appendices, Appendix C discusses the position of archaeologists in ancient DNA research and the information disconnect that exists between published calls by molecular researchers to archaeologists urging them to become more involved in ancient DNA research projects and the archaeologists who are the target of such calls. While this information is not essential to this study, it is nonetheless important, and as such had been included in the Appendices as supplemental information. Appendix C is composed of three parts. Part One discusses the development of archaeological methodology from an historical perspective. Part Two discusses the information disconnect between molecular researchers and archaeologists. Part three provides a discussion of the archaeological prerogative as the point of first contact with samples and their specialized skills for acquisition of such samples. A model of an idealized excavation for down the line aDNA applications is also included. This study propels both archaeoparasitological research forward as well as identifying areas of weakness which hamper both archaeological and modern molecular parasitological work.

Chapter Two: Background Information

La Cueva de los Muertos Chiquitos, El Zape, Durango, Mexico

The archaeological site of *La Cueva de los Muertos Chiquitos* (Cave of the Dead Children) is located above the Rio Zape as it blends into the Rio Sestin river system just north of El Zape, Durango, Mexico (Figure 2).

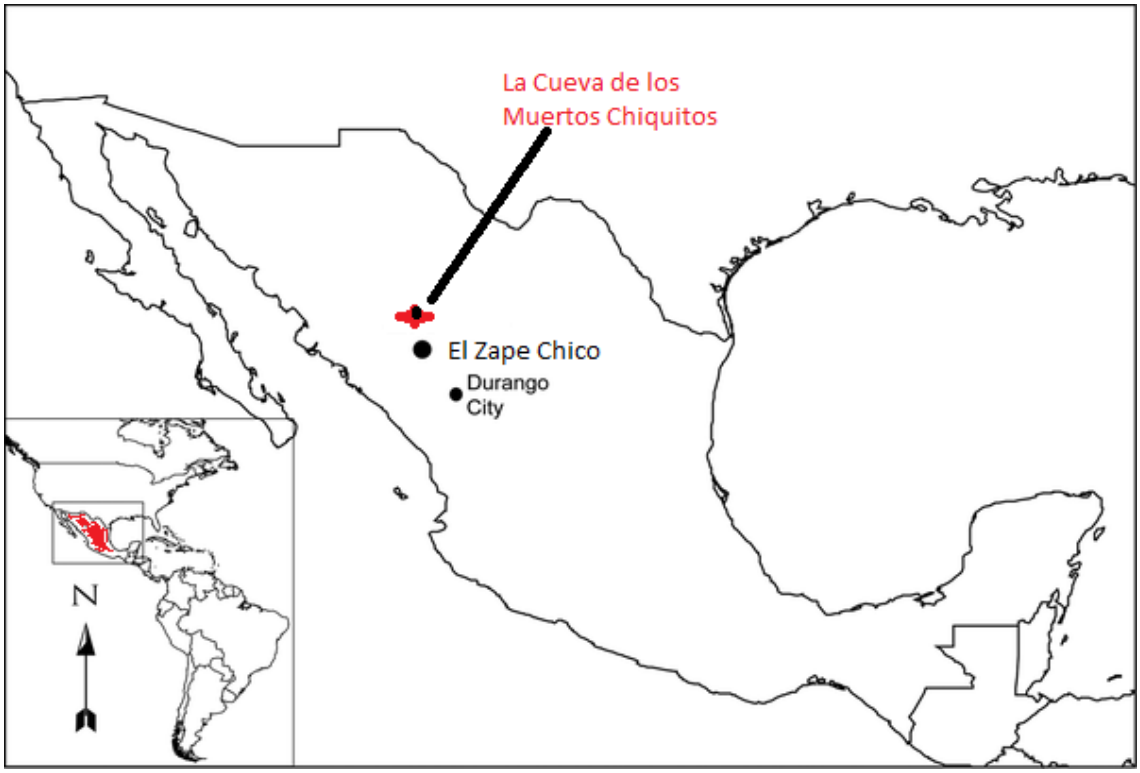

Figure 2: Location of *La Cueva de los Muertos Chiquitos* archaeological site adapted from (Tito, et al. 2008)

The site was located during large scale survey of the eastern foothills, of the Sierra Madre Occidental mountain range, of the Zape region by Dr. Richard Brooks in the 1950s (Brooks, et al. 1962; Foster 1978, 2000; Tito, et al. 2008). The site is in a cliff face. A trail runs from the town of El Zape Chico to destinations north and runs about eight feet below the cave entrance. The

remaining vertical eight feet to the cave are accessed by finger and toe holds. The cave itself is approximately 9 meters (30 feet) deep and 18 meters (60 feet) wide. The use of adobe to create puddled floors and partitions differentiating interior cave space indicates cultural renovation of the cave interior (Brooks, et al. 1962). Renovation coupled with the midden and cultural artifacts, including floral and faunal remains, basketry, pottery, shell beads and ornaments, and lithic tools, supports that this site was a habitation site (Brooks, et al. 1962)

La Cueva de los Muertos Chiquitos, is so named because of the discovery of a number of child burials within the cave, sealed beneath puddled adobe floors (Brooks, et al. 1962; Brooks and Brooks 1978). John Crandall has identified 31 individuals from the site, with 19 of them being children younger than ten and a large percentage of those being very young children (Crandall and Thomson 2014; Crandall, et al. 2012). The earliest Radiocarbon analysis on a piece of wood recovered from beneath the puddled adobe floor in section B4 (Figure 3) places the earliest date of occupation at AD 600 (1300 +/-100 B. P.) (Brooks, et al. 1962). Additional dates from the later burials indicates a date of AD 1168. Crandall and his colleagues place the span for the burials as AD 571 – AD 1168. Pottery styles provide a terminal age of AD 1150 (Crandall and Thomson 2014). This places the people at *La Cueva de los Muertos Chiquitos* as occupying the cave contemporaneous with the Mesoamerican Classic era AD 200 – AD 1000, and perhaps straddling into the Post Classic Era. Brooks originally suggested the site was occupied from about AD 600 until about AD

1450, however, the newer dates seem to narrow occupation to no later than AD 1200.

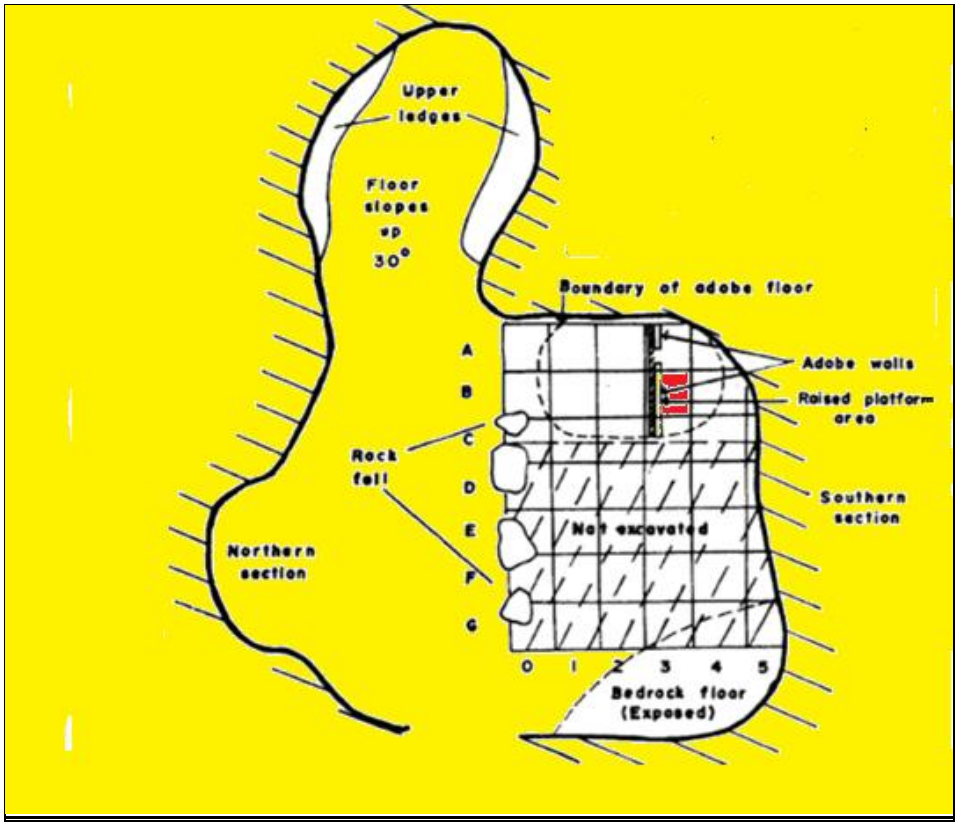

Figure 3: Map of the Interior of *La Cueva de los Muertos Chiquitos*, Showing Area of 1957 Excavation. Unit B4 is Marked in Red. Adapted from the Map by (Brooks, et al. 1962).

The Sierra Madre Occidental mountain range runs from the south-central part of Mexican state of Chihuahua north into the north-central Mexican state of Durango. The Zape region is located securely in the geographical foothills zone of the El Norte Mesa. The area is generally a series of abrupt mesas and hills rising in some cases 60 meters (200 feet) from the basin-range country of the lowland. It has been variously described as a mesothermal savannah and a hot

steppe environment, with generally oak and mesquite grasslands (Foster 1978, 2000).

The area receives 500 – 700mm (20 – 28 inches) of precipitation each year, with the wet season coinciding with growing season, making dry farming possible (Brooks, et al. 1962; Foster 1978). The Rio Zape generally runs year round and the area has an average temperature of between 12-18 degrees Celsius (53 -65 degrees Fahrenheit) (Brooks, et al. 1962). The cooler months can reach lows of -3 degrees Celsius (26 degrees Fahrenheit) but range up to 18 degrees Celsius (65 degrees Fahrenheit) creating rather mild winters. Precipitation for the dry months can be less than 40mm (2 inches). Drought during the summer can lower agricultural production, and even further limit agricultural production if the dry month precipitation is exceptionally low (Foster 1978). Regardless, the area has sufficient moisture to permit dry farming and supports a range of natural resources available for human exploitation.

Botanical resources available in the area include: piñyon (*Pinus sp*.), juniper (*Juniperus sp*.), cypress (probably *Cupressus sp*.), madrono (*Arbutus sp*.), manzanilla (*Malvaviscus sp*.), several varieties of agave (*Agave sp*.) and cacti (*Opuntia sp*.), a sunflower like plant called *Tithonia*, at least ten species of oak (*Quercus* spp.) with edible acorns, walnut (*Juglans* spp.) and especially in the Zape region, mesquite (*Prosopis sp*.), river poplars (*Populus spp*.), and willows (*Salix spp*.). The faunal assemblages include: coyote, rabbit, squirrel, mice, rats and other rodents, deer and possibly mountain sheep. Three types of fish and turtle were also exploited (Brooks, et al. 1962; Foster 1978). The

position of the Zape region allowed for vertical resource exploitation, gathering animals and other material like mushrooms from higher elevations, as well as utilizing the rich-soiled valley bottoms for planting of domesticated foods, such as, multiple varieties of beans and corn as well as squash and gourds (Foster 1978). Despite its subhumid, temperate clime amidst more arid areas, the Zape region was an area with an abundance of resources allowing for the sustenance of human populations and this area was the primary zone of occupation for prehistoric peoples (Foster 1978).

Based on material culture and the location of the cave, the site has been assigned to the Loma San Gabriel culture, without Chalchihuites influence. The Loma San Gabriel culture is known from the type site, the Weicker site, described by Foster (1986). Good chronological and cultural knowledge of this area is extremely limited (Foster 2000). There are approximately 50 sites attributed to this culture, but they are known mostly from surface collections and three partial excavations (Foster 1978). The Loma San Gabriel people are thought to have occupied the area from AD 100 to AD 1450 (Brooks, et al. 1962; Foster 1986). Sites extend from the Rio Conchos drainage in southern Chihuahua to Durango and west Zacatecas. Three other cultures are also present in this area during the same period - the elaborate Mesoamerican oriented culture of the Chalchihuites, known from the Alta Vista site, the Malpaso known from the La Quemada fortress site and the Bolaños, which represent an archaeological zone in south Durango and north Jalisco, with

evidence of extensive trade between long distance partners from the highlands to the coastal regions (Foster 1978).

When Charles Kelley began surveys and excavations of the area in the 1950s, his intent was to identify the northwestern boundary of Mesoamerican culture. Additional archaeology by southwestern archaeologists attempted to determine the southern extent of the Hohokam and Mogollon cultures, but had little interest in making connections with Mesoamerica. Richard Brooks conducted additional surveys of the Zape region, which led to the discovery of the *La Cueva de los Muertos Chiquitos* site (Brooks, et al. 1962; Brooks and Brooks 1978; Foster 1978).

Despite the paucity of evidence in the area, it has been argued that the Loma San Gabriel culture grew from the local Archaic population, which itself grew from earlier Paleo groups in the same area. It is argued that the Loma San Gabriel groups may have been ancestral to the modern Tepehuan and Huichol groups (Foster 1978, 2000). Crandall, et al.(2012) associated the Loma San Gabriel as Tepehuan, rather than an ancestral group. Crandall and Thompson (2014), interpreted the child burials at *La Cueva de los Muertos Chiquitos* as infant sacrifices, associated with the Mesoamerican Tlaloc Ceremonial Complex. They base this sacrificial interpretation on a turtle effigy recovered from the site and the diseased state of many of the infant burials. Based on a Colonial account of Contact Era Tepehuan infants were sacrificed during disease outbreaks in an effort to save other members of the group. Brooks, et al (1962) also suggested the original set of infant burials might have been

sacrificial victims for the house construction. However, Foster (1978) later suggested the burials from beneath the oldest section of adobe floor were the victims of a disease episode, which killed swiftly and struck the youngest members of the social group. Jiménez, et al. (2012) and Cleeland, et al. (2013) both suggest parasitism as a cause of both morbidity and mortality among the infants buried beneath the oldest floor. Sacrificial associations are problematic for a number of reasons, not the least of which is extrapolating back cultural practices greatly removed from the context of the Loma San Gabriel culture, which is definitively simple in presentation and possesses no overt artifacts of religious association either in icongraphy or architecture.

As mentioned above, the Loma San Gabriel material culture is rather utilitarian in comparison to the Chalchihuites material culture and the more complex Mesoamerican cultures to their south. It consists of two utilitarian pottery types, known as, Loma Plainware with surface colors from buff to white, and Loma textured, which had a scratched surface, and one decorative pottery style, Chico Red on Brown pottery that may or may not have been an attempt to replicate Chalchihuites pottery (Foster 1978). Chico Red on Brown is crudely polished and decorated. Some sites show intrusive Chalchihuites pottery styles or poor replicas (Foster 1978). Lithic tools are not exceptionally abundant and are crudely made, although at Zape an obsidian knife and a chert lance head were more finely knapped (Brooks, et al. 1962). Groundstone at the site showed no evidence of preparation by shaping before use, but was modified by use (Foster 1978). There is evidence of basketry and weaving, and cotton fibers

have been identified from the cave (Brooks, et al. 1962; Brooks and Brooks 1978; Foster 1978).

Settlement patterns among the Loma San Gabriel were variable, meaning the placement of houses and the type of houses showed no distinguishable pattern. Generally, the sites consisted of small hamlets or villages, on non-arable land situated on the mesa tops or for those in the Zape region in caves and rock shelters in the cliff faces (Foster 1978, 1986, 2000). These sites were above arable land and were defensible. A few sites may have had a central plaza associated with a mound and organized house patterns, but on the whole no central unifying structure and no ceremonial structures have been identified. House types have been rectangular, single to multi-room houses, with rock or adobe brick foundations and round structures of wattle and daub. Some houses were also encircled by short rock walls. Some houses contained paved floors, while others were packed earth. (Foster 1978, 1986).

The greatest variety and complexity in Loma San Gabriel material record occurs in the numerous shell beads and pendants at the site, many associated with the burials (Brooks, et al. 1962; Brooks and Brooks 1978). The source material is marine, suggesting long distance trade with coastal groups. One type of reed found at the *La Cueva de los Muertos Chiquitos* site is also non-local and associated with the coastal area (Brooks, et al. 1962). Thus at least two lines of evidence support coastal trade.

The Loma San Gabriel subsistence shows a mixed style relying on the domesticated crops of corn, beans and squashes, while also heavily exploiting

wild plant materials including pine nuts, black walnuts, acorns, agave, cactus, and sotol. Wild game and fish provided protein, and included coyote, jackrabbit, squirrels, mice and rats, as well as larger mountain sheep and deer (Brooks, et al. 1962; Foster 1978). It is also possible, and has been suggested, that domesticated dogs and turkeys may have also been a part of the subsistence system (Foster 1978).

To the south, at this same time, Mesoamerican groups were raising a variety of hairless dog, specifically for food. The Tula Hidalgo site, dated from AD 650- AD 750, provided evidence of three well defined canine species, at least one of which was used for food - the traditional medium sized Mesoamerican dog, with hair, the *itzcuintli,* a medium sized hairless known as the *xoloitzcuintli,* and a small hairless known as the *tlalchichi* (Azua, et al. 1999). This latter dog is believed to be immortalized in Colima pottery, often shown with corn in its mouth. These same species were described by the 16th century Spanish chroniclers (Azua, et al. 1999). Schmitt (1952) noted that the Mexican hairless dog was particularly desirable for boiling and that puppies of all breeds were preferred over older dogs because their meat was the most tender. Numerous accounts of early Europeans, noted that the use of dogs for food was widespread across the tribal groups of the Americas, even if not found in all groups, and that dog breeding was common (Lallemant 1901a, 1901b; Lambourville 1901; Levanthal, et al. 2012; Maximillian 1906). Larger dogs were bred for transport and hunting, while smaller dogs were bred for food. Even

when not bred specifically as a food source, in times of inadequate food, dogs were used as a food source (Kerber 1997).

The exploitation of insects, especially ants, grasshoppers, crickets, beetles, and moths may have also provided protein for the inhabitants of the Zape region. Providing excellent protein and other essential vitamins, the collecting of insects is a common practice worldwide and prehistoric evidence suggests entomophagy (ingestion of insects) was a common practice in northern Mexico (Callen 1965; Gahukar 2011; Itterbeeck and Huis 2012; Ramos-Elorduy 2009; Sutton 1995).

A large number of human coprolites and quids of chewed fibrous plant materials have also been recovered from the site. Human coprolites used in this study were located beneath a puddled adobe floor, which acted to enhance preservation of not only the coprolites but other cultural material.

In summary, the inhabitants of *La Cueva de los Muertos Chiquitos* have been assigned to the Loma San Gabriel culture. This culture may have been ancestral to the modern Tepehuan groups. The cave inhabitants utilized a mixed subsistence strategy that included dry farming of a variety of corn, beans and squash, coupled with heavy exploitation of the natural resources of the area. Hunting provided protein from a variety of mammals and fish still available in the area today. The Loma San Gabriel people lived in a variable settlement pattern that made use of non-arable caves, rock-shelters, and mesa tops for habitation, while exploiting the fertile, arable land in the river bottoms for crops. Their material culture is relatively plain and functional. The occupants of the

caves and rock-shelters transformed the interior space using adobe brick and puddled flooring for human habitation. The burials of a large number of young children, in at least two separate events, require serious study and resolution, requiring a focus on the health risks and factors within this prehistoric population. This study seeks to examine whether molecular methods can provide additional unique data to assist in defining the role of parasitism in this prehistoric population and interpreting the possible health implications for the inhabitants of *La Cueva de los Muertos Chiquitos*.

DNA Sequencing: Sanger and Next-Generation Technology

The classical genetic sequencing method is called Sanger sequencing often utilizing a capillary reading system of 96 well plates, where each well ideally represents a single DNA sequence read. Each well is read individually. This method is limited to the number of sequences it can process in a single batch and provides read lengths of generally ~650-800 but can reach up to 1200 nucleotide base pairs (bp) of DNA (Mardis 2007; Zhang, et al. 2011).

Next-Generation sequencing (NGS) technology provides short reads (short fragments of genetic sequence), much shorter than the typical Sanger sequence, but provides far more data return, reducing overall costs of sequencing. Read length, however, is important and should be considered when choosing a sequencing method. For example, Wommack, et al. (2008) compared longer reads to subsets of shorter reads derived from the longer reads and found that short reads failed to find homologs (similar gene sequences due to descent from a common ancestral DNA sequence) more

often than longer reads when submitted for assignment. This failure was especially evident if the homolog of the longer read was more distant taxonomically. Short reads also provide less information in relation to larger reads and may entirely fail to match functional gene sequences. These results led them to suggest that the failure of short reads might make them inappropriate for characterizing microbial communities. This may make them even less suitable to identifying the less frequent components of a mixed sample.

Despite the difficulties of short reads they are useful in aDNA studies, addressing gaps in whole genome sequencing and in the sequencing of an artificial bacterial chromosome (BAC) that carries and stores genetic information or a fosmid, which is a DNA construct, which also carries low copy genetic material and stores it. Both, BACs and fosmids are used as vectors for cloning large numbers of genetic clones in bacterial studies (Tito, et al. 2008; Valentini, et al. 2009; Wommack, et al. 2008). Short read lengths are also appropriate in targeted studies when the target area is highly informative between species and less than 200 bp in length, such as the 12S and 16S genes of the mammalian mitochondrial genome (Karlsson and Holmlund 2007). Using small variable sections of the 12S or 16S rRNA genes allows for the identification of species, as well as primers that capture small enough targets to capture fragmented and degraded DNA.

Next generation sequencing systems have increased the processing power and the speed of sequencing ten to one hundred fold. At this time, a

single next generation batch is much more expensive than a single batch of Sanger sequencing, which can limit its feasibility if a project has limited research funds. However, relative to the number of base pairs obtained, next generation technology is the far more economical choice for the amount of data obtained. Researchers should take into consideration their information needs, budget, and the differential utility of all sequencing options when designing their research plans (Kunin, et al. 2008; Wooley, et al. 2010).

There are several good reviews available comparing Next-Generation Sequencing Platforms and discussing Next Next-Generation technologies which began reaching the market in 2011, such as the much anticipated Single Molecule Real Time (SMRT) platforms (Harismendy, et al. 2009; Kunin, et al. 2008; Quail, et al. 2012; Zhang, et al. 2011). SMRT platforms can extend the read lengths with the potential to surpass the lengths obtained through Sanger sequencing.

The three Next-Generation sequencing platforms that have dominated the market are the Roche 454 Pyrosequencer, the Illumina GA sequencer, and the ABI SOLiD sequencer. Harismendy, et al. (2009) reviewed these three systems in comparison to traditional Sanger sequencing results. They found all three systems provided high sensitivity with a greater than 95% accuracy of variant calling. A variant differs from the reference sequence by one or more nucleotides at a given location. Coverage is defined as the number of times a particular spot on the sequence has been repeatedly captured during sequencing. They found that at high coverage depth, meaning each nucleotide

being represented multiple times, base calling errors were systematic across all platforms and were directly related to the sequence context. Sequence context can be thought of as the topology of the sequence and includes variables such as insertion/deletions, repetitive sequences, GC and AT composition percentages. Nucleotides are generally found in linked pairs, G and C complement each other while A and T complement each other. A large percentage especially a GC rich sequence can be difficult to sequence. Random base errors are encountered with lower coverage. An additional concern they noted was the discrepancy between coverage depth between overlapping ends and the actual body of the sequence for Illumina GA runs, resulting in up to 56% of the reads associated with the overlapping ends. Illumina chemistry uses paired ends that match up, as a result the study found that a larger percentage of the reads were associated with the overlapping ends, rather than independent reads. The chemistry introduces a bias in the reads recovered due to the end pairing steps, which results in a higher percentage of reads being associated with the paired ends and not the sequence targets. They suggest trimming the ends. In contrast, the 454 platform only had about 5% overlap. Overall, this study found that 454 sequencing provided the most even coverage for problematic areas such as unique variants or repetitive areas. Illumina GA returned the most variability and the ABI SOLiD returned a strong bias against covering repetitive areas.

Two years later, Zhang, et al. (2011), reviewed the five platforms currently on the market, noting that the market was dominated by versions of

the original three platforms: Roche GS-FLX 454 Genome Sequencer, the Illumina/Solexa Genome Analyzer and the ABI SOLiD sequencer. The three original systems used sequencing by synthesis or sequencing by ligation technologies. Sequecning by synthesis uses polymerase to build a new molecule of DNA from the template and captures the florescent signal released as each new nucleotide is added to the string. Sequencing by ligation, also produces a chemical signal, but uses ligase which acts as a glue to attach matching nucleotides and then cleaves them away for another round. This results in each subsequent read being one or more base pairs shorter than the previous read. Of the two newer platforms, the Polonator G. 007 system also used sequencing by ligation, while the Helicose HeliScope system was the first SMRT technology and is considered next next-generation sequencing. Also, Glenn (2011) published “Field Guide to Next Generation DNA Sequencers” in Molecular Ecology reviewing six systems currently available. His tables of recommendations are updated annually and are housed on the Molecular Ecology website located at: http://www.molecularecologist.com/next-gen-fieldguide-2014/.

Two platforms designed for desktop use in small laboratories were released in 2011, these include the Illumina MiSeq platform and the Ion Torrent Personal Genome Analyzer (PGM). The Pacific Bioscience’s commercial SMRT technology RS sequencer was also released in 2011. Quail, et al. (2012) tested and reviewed these three systems in comparison to the current market leading system of Illumina’s HiSeq system. Sequence contextual errors continue to

cause issues for both the Illumina MiSeq and the Torrent PGM with the PGM specifically unable to sequence AT rich areas and while the Torrent PGM was able to call slightly more variants than the MiSeq it had a higher false positive rate. The Pacific Bioscience RS system required very high coverage for variant calling. The MiSeq system takes an average of 27 hours to process a run, while both the Torrent and RS systems require only two hours. The Torrent returns reads of about 200 bases, whereas the MiSeq provides average read lengths of up to 150 bases, the same as the Illumina GAIIx and the Illumina HiSeq platforms, while the RS platform returns larger read lengths that include both adapters, which need to be trimmed away and reverse strand sequences.

In 2013, the FDA authorized the Illumina MiSeqDX for use in clinical laboratory settings, marking the first FDA authorization of a next-generation sequencing platform (Collins and Hamberg 2013). Next generation technology has been and will continue to rapidly evolve. As a result the read lengths become longer and the accuracy more improved. And, as the technology moves to versions acceptable for use in smaller laboratories and clinical settings, the use of next generation high throughput technology opens the doors to increased experimental and diagnostic use at ever lessening costs per run.

Because of the enormous amount of data recovered from next-generation sequencing, bioinformatics issues remain (Kunin, et al. 2008; Pop and Salzberg 2008). In an effort to alleviate this, software is available from multiple sources, including software packages provided with the systems, as well as publicly available methods (Altschul, et al. 1990; Bandelt, et al. 2004;

Bhatia, et al. 1997; Gelbert 1998; Meyer, et al. 2008; Tamura, et al. 2011). Even so, the time and the processing of the data after the fact is hampered by a number of issues, not the least of which, is an inadequacy in the currently available databases, especially in relation to non-bacterial studies. These issues are not insurmountable, but it is necessary for researchers to highlight issues, design studies that provide their own reference material for comparison and for the sciences to work to bolster the current databases with additional genomic sequencing information for non-bacterial organisms. These issues will be more fully discussed in the results for this chapter.

While there are multiple platforms available, of importance to this study is specifically the earlier 454 pyrosequencing, which uses a sequencing by synthesis process. This platform requires the building of DNA libraries and the use of adapters to tag reads and also allows for either the whole genome shotgun sequencing method or a targeted specific loci method. The shotgun method was developed during the Human Genome Initiative (Zhang, et al. 2011). Shotgun sequencing works by synthesizing small fragments of DNA to later be reconstructed into whole genomes – or mapped to reference sequences. Targeted sequencing begins with the targeted amplification of a particular genomic location, for example, 16S rRNA for bacteria or 18S rRNA for eukaryotes. The development of bar-coding or indexing tags allowed for the pooling or multiplexing of several samples into one run. The tags identify with which sample a read is associated, allowing for the examination of multiple

samples and thus lowering overall sequencing costs even further (Parameswaran, et al. 2007).

Ancient DNA Technologies: PCR and NGS

The earliest ancient DNA studies utilized traditional cloning procedures. Cloning is a method of amplifying genetic template encapsulated into a plasmid (mobile DNA element) inside a modified bacterial cell, which captures a single genetic fragment, the bacterial cells are then cloned in a culture medium, usually an agar – which is a nutrient gel conducive to bacterial replication. As the bacterial clones replicate, colonies become visible on the agar plate and can be removed with a pipette. Ideally, these colonies will each contain enough of the DNA templates for analysis through sequencing (Strachan and Read 1999). There are only two published aDNA articles using this technique, the amplification of extinct quagga DNA (Higuchi, et al. 1984) and the amplification of DNA from an Egyptian mummy (Pääbo 1985). Cloning is still employed in order to replicate sequences for verification and to sort out mixed samples, but is no longer the primary method for aDNA work. The majority of aDNA work has used Polymerase Chain Reaction (PCR) technology developed in 1986 by Kary Mullis (Kelman 1996; Mullis and Faloona 1987; Mullis, et al. 1986; Saiki, et al. 1988).

PCR revolutionized many fields of biology, not the least aDNA analysis. The process uses polymerase, an enzyme responsible for DNA replication found in all living cells. A heat stable polymerase was necessary so that the polymerase would not become inactive at temperatures necessary to denature

double stranded DNA. A polymerase was isolated from *Thermus aquaticus,* a thermophilic or heat loving bacterium found in geysers and deep sea vents, where the temperatures were once thought to prohibit life (Saiki, et al. 1988). Because this particular polymerase can withstand high temperatures, it retains its active function during the variable thermal cycling of the PCR technique. This technique amplifies low copy number and small fragment size DNA from modern or ancient samples. Using primers to anneal to each end of the targeted section of genetic sequence desired, the PCR technique makes millions of copies called amplicons, by proceeding through a series of steps at different temperatures that separated the double stranded DNA into two strands. The primers which are short tags of oligonucleotides that match a segment of the desired sequence, attach on the 5' prime ends of the separated strands and reads toward the 3' prime end (see Figure 4). At the proper temperature the free nucleotides will be incorporated into the single strands by the polymerase. This series of steps is repeated generally 30 to 60 times per reaction, exponentially doubling the DNA sequences with each cycle (see Figure 4). Primers are short sequences of 25 nucleotides or less, which flank the desired sequence being targeted. They attach to single stranded DNA and then polymerase incorporates the sequence to create a new double stranded molecule. Primers can be for a specific organism and are called species specific primers, or they can be general, meaning they will find and attach to the same sequence section in multiple organisms. Newer sequences are experimenting with blocking or bait primers, which when used in conjunction

with other primers, act to capture the majority of sequences for which they have been designed, for example mammals or bacteria. Blocking primers are then ended with a blocking sequence that prevents that sequence from being replicated. This is a good method for removing high number sequences which may inhibit the recovery of rare sequences in a sample. It allows for the enrichment of rare sequences via PCR.

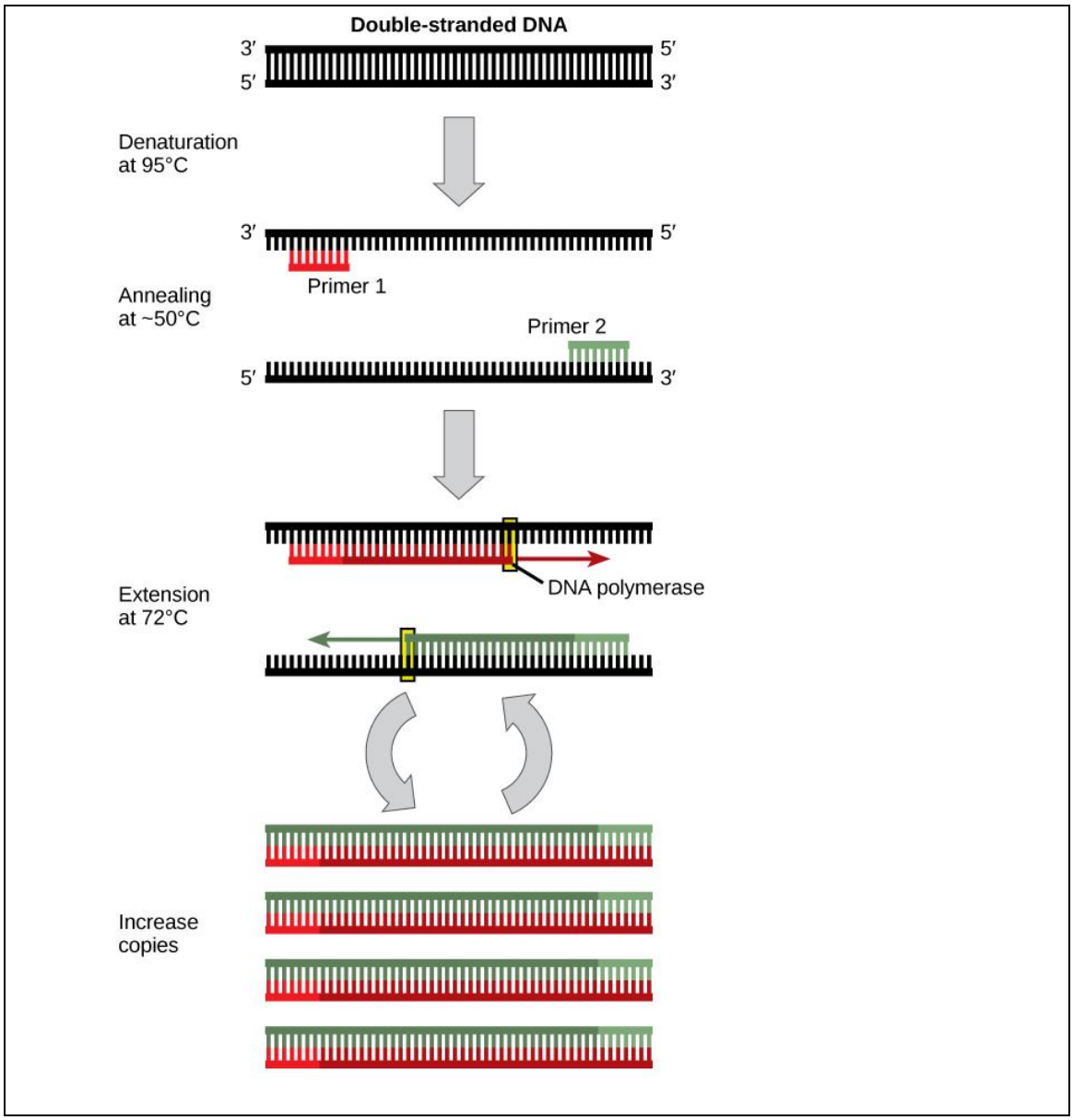

Figure 4: PCR amplification. Open Source Image from http://cnx.org/resources/f53a4f383b883f06470bc72c2f8434df/Figure_10_01_03.jpg

PCR produces enough copies of a template sequence for the material to be visualized by electrophoresis and sequenced for additional analyses, such as taxonomic identification or phylogenetic tree building. The limitations of PCR include because it is highly sensitive and can amplify low copy template, it will

also preferentially amplify the most abundant DNA in a sample, which is often contaminate modern DNA when working with degraded ancient samples. Mixed samples that include the genetic material of multiple organisms all having the same targeted sequence, will confound sequencing and must be cloned to separate the different samples. Inhibitors from the original sample can be co-amplified and prevent DNA from being isolated. While many PCR reactions can be made, they are generally a single sample per reaction. This can become labor intensive and costly. Nevertheless the majority of aDNA studies have utilized PCR. Pääbo, et al. (2004) published a good review of aDNA research in 2004, followed by Willerslev and Cooper (2005). Knapp and Hofreiter (2010) published an excellent review of aDNA and Next Generation Sequencing (NGS). Rizzi, et al. (2012) published another excellent review which discusses PCR in the context of the classical methodology of aDNA research and provides an extensive discussion of the new methodology which includes NGS applications and future prospects. Matisoo-Smith and Horsburgh (2012) have published a book targeted toward archaeologists and aDNA analyses.

The early aDNA studies brought to light some of the serious difficulties inherent in the science, for example, claims of DNA recovery from Miocene fossils (Golenberg, et al. 1991) or amber encrusted organisms (Cano, et al. 1993; DeSalle, et al. 1992), were unable to be replicated by later studies and determined to be contaminate sequences rather than authentic aDNA (Austin, et al. 1997). This led to a number of stringent criteria of authenticity (see Table 1) being published, which were to guide and be the standard by which all aDNA

studies were evaluated (Cooper and Poinar 2000; Gibbs 1993; Handt, et al. 1994; Michael Hofreiter, et al. 2001; Kemp and Smith 2010; Pääbo 1989; Pääbo, et al. 2004; Poinar 2003).

Table 1: General Criteria of Authenticity based on a version from Poinar (2003) and modifications from Kemp and Smith (2010).

Criterion	Comments
Dedicated and Isolated work area	Lab should be a one way work flow lab, physically isolated from any lab performing modern DNA work and used only for aDNA work.
Controls samples for each step: Extraction -- Blanks PCR -- Blanks Positive Controls	Blanks are added to each step and processed in parallel with the aDNA. Positive controls are created in the modern lab and added to single tubes in the modern lab, to prevent cross contamination.
Molecular Behavior should be appropriate	Amplication strength when visualized should be greater the smaller the fragment. Generally aDNA will be less than 300bp.
Quantiation – Real Time PCR **	This is to test the amount of template available. The use of fluorescent Real Time PCR allows aDNA to be quantified and can be watched in real time. Other methods such as Nano Drop or Bioanalyzers are also possible to use, but may not provide the best results.
Reproducibility	Multiple extractions and PCRs should be performed for each sample, showing the same results consistently.
Cloning **	Verify the direct PCR sequencing results by also sequencing a number of clones via bacterial cloning.
Independent Replication **	Can be done in independent labs or can be performed by different personnel in the same lab.
Biochemical Preservation **	Testing the preservation of other proteins led to the suggestion of using amino acid racemization to determine the preservation of other biomolecules, which would suggest the preservation of authentic aDNA.
Associated Remains **	Associated remains such as faunal remains may be the most conservative test of preservation. If aDNA remains in associated remains, aDNA can be assumed to be present in human remains. Also important contextually, as it could provide a point of contamination.
Phylogenetic Sense	The recovered sequences need to make sense. If a human sample was used, then the sequence should not come back as a horse.
** conditions that can be modified today	

This also led to a split in the field, one branch focusing primarily on the development of methods (Collins, et al. 2009; Gilbert, et al. 2005; Hagelberg and Clegg 1991; Handt, et al. 1994; Pääbo 1989; Yang and Watt 2005) and the other with applying aDNA to actual studies (Borson, et al. 1998; Briggs, et al. 2009; Cleeland, et al. 2013; Deagle, et al. 2009; Gilbert, et al. 2008; Green, et al. 2008; Green, et al. 2009; Guhl, et al. 1999; Hebsgaard, et al. 2009; Krause, et al. 2010; Leles, et al. 2012; Li, et al. 2000; Loreille, et al. 2001; Losey and Yang 2007; Oh, et al. 2010 ; Pääbo 1985; Rasmussen, et al. 2010; Tito, et al. 2011; Tito, et al. 2012; Tito, et al. 2008; Willerslev, et al. 2003).

Studies of ancient remains have been successfully conducted with a variety of remains, hair (Amory, et al. 2007; Gilbert, et al. 2008; Gilbert, et al. 2004; Rasmussen, et al. 2010); bone and teeth, including Neanderthal and Denisovian individuals (Cannon and Yang 2006; Green, et al. 2009; Green, et al. 2008; Pruvost, et al. 2007; Reich, et al. 2010), dental calculus (Adler, et al. 2013; Preus, et al. 2011; Warinner, et al. 2014a; Warinner, et al. 2014b), mummified tissues (Fletcher, et al. 2003; Guhl, et al. 1999; Pääbo 1985), coprolites (Cleeland, et al. 2013; Gilbert, et al. 2008a; Iñiguez, et al. 2006; Iñiguez, et al. 2003; Leles, et al. 2012; Loreille, et al. 2001; Oh, et al. 2010 ; Tito, et al. 2011; Tito, et al. 2012; Tito, et al. 2008) and sediments (Hebsgaard, et al. 2009; Malmström, et al. 2009; Willerslev, et al. 2003). As noted earlier, this is by no means exhaustive and good reviews are available for more information. Some studies, such as the Neanderthal, Denisovian and

microbiome studies using dental calculus and coprolites have been among the most fruitful studies using Next Generation technology discussed below.

The advent of Next-Generation sequencing technologies have provided enhanced abilities to process large organism rich samples, such as soil, compost and microbiome samples from the gut, via fecal or coprolite samples and samples of other body locations. These technologies provide a tremendous amount of data which presents some bioinformatics difficulties for the management of such large datasets (Mardis 2007; Pop and Salzberg 2008). However, several computer programs and information management systems are now in place, such as the Metagenome RAST server, which processes raw 454 DNA sequences and returns information on thousands of small genetic fragments, using interfaces with a number of databases to provide possible species and gene identification (Meyer, et al. 2008; Wooley, et al. 2010). While the majority of studies have involved modern samples, Next-Generation sequencing has also been applied to microbiome studies of ancient coprolite samples, providing excellent bacterial classification of ancient gut biomes (Tito, et al. 2012; Tito, et al. 2008; Yang and Watt 2005). Next-Generation technology has also been used to characterize the entire genomes of extinct Hominid species, such as Neanderthal and the recently characterized Denisovian hominid found in Denisova Cave, Siberia and Paleo-Eskimo and pre-Clovis individuals via coprolite material (Green, et al. 2009; Green, et al. 2008; Krause, et al. 2010; Rasmussen, et al. 2010; Reich, et al. 2010).

Because ancient DNA is highly fragmented and of small size, it should be ideally suited to Next-Generation sequencing technology and as noted above has been successful in both whole genome shotgun approaches such as those used for the characterization of microbiomes and environmental samples, as well as, in targeted approaches that seek specific organisms such as prey animals or Neanderthal DNA (Briggs, et al. 2009; Deagle, et al. 2009; Gilbert, et al. 2008a; Gilbert, et al. 2008b; Green, et al. 2008; Karlsson and Holmlund 2007; Knapp and Hofreiter 2010; Krause, et al. 2010; Malmström, et al. 2009; Poinar, et al. 2006; Rasmussen, et al. 2010; Tito, et al. 2012; Tito, et al. 2008; Valentini, et al. 2009).

For organisms that represent a small percentage of a larger sample, targeted sequencing is a more productive approach, however, this is not always possible. Attempts at a targeted 18S Illumina run for this study were not successful. However, it is possible that within those reads assigned originally to the eukaryota would be reads identifiable to the other non-bacterial components of the sample, such as the host, foods consumed, both plant and animal, as well as any parasites that may be within the host (Carpenter, et al. 2013). This study, therefore filters and analyzes two complete 454 pyrosequencing datasets for Zape 23 in an effort to identify parasites within the coprolite material.

Coprolites

Coprolites were first considered for analysis by Dr. Jonn Harshberger in 1896 (Heizer and Napton 1969; Patrucco, et al. 1983). Harshberger suggested that studying the material inclusions within coprolites would offer insight into

prehistoric diets. Clinical protocols to examine modern feces by rehydration started around 1898 (Heizer and Napton 1969). Col. Bennett Young examined the remains from Mammoth and Salts Cave and published in 1910 the results of a dry study revealing sunflower seeds and hickory shell fragments (Heizer and Napton 1969). In 1912, L. L. Loud reported on the broken coprolites of suspected human origin from Lovelock Cave, Nevada and described their dietary content. This research offered potential for expanding direct knowledge of the diets of prehistoric humans and animals, but was not pursued until the examination of ground sloth coprolites for diet in the 1930's (Heizer and Napton 1969).

Bryant and Dean (2006) provide an excellent history of coprolite science in their tribute to E. O. Callen, a botanist by training, who became the founder of archaeological coprolite analysis. Especially important in their article, is a discussion of the early treatment of coprolite material as a non-informative and annoying artifact, which were more often than not, destroyed rather than collected.

In 1951, Junius Bird, an archaeologist excavated the Huaca Prieta Chicama site in Peru. He sought the assistance of T. W. M. Cameron, a parasitologist, at McGill University, in Canada, to examine the desiccated fecal material for parasites. E. O. Callen, a botanist, also at McGill University, learned of the samples and asked to examine a few for a fungal study he was interested in pursuing. The collaboration that followed provided the impetus and foundation for all subsequent coprolite studies. Immediately apparent to the two

McGill University researchers was the need for an effective method for rehydrating the samples without damaging their delicate inclusions (Bryant and Dean 2006; Callen and Cameron 1955). Based on a protocol developed by van Cleave and Ross (1947) for rehydrating desiccated tapeworm samples in the lab, Callen and Cameron developed a 0.5% trisodium phosphate rehydrations protocol (Callen and Cameron 1955, 1960). Callen continued to refine the protocol over the next decade (Callen 1963, 1965). Callen's protocol has become the standard for coprolite analysis, however, it contains formalin and formalin can cause issues with DNA. Therefore some molecular researchers have opted for alternative rehydration solutions such as water, Tris-EDTA (Cleeland, et al. 2013; Iñiguez, et al. 2006; Iñiguez, et al. 2003) and/or glycerol (Loreille, et al. 2001).

Despite the earliest cursory examinations of dry coprolites, the science did not become an active and viable subject until Callen and Cameron developed the rehydration protocol (Bryant and Dean 2006; Callen 1965; Callen and Cameron 1955). Callen switched his focus to archaeological samples and spent the remainder of his time developing this science in relation to both dietary elements and parasites (Bryant and Dean 2006). As this study is focused on parasites, the discussion will be narrowed to those topics only.

Coprolites and Parasites

Initial coprolite studies for parasites involved rehydration and flotation of samples to concentrate parasite eggs and then preparation of microscope slides for viewing. Studies using this approach were fruitful, but with inherent

limitations. A 1969 analysis of coprolites from Danger Cave, Utah, was unable to determine which species of *Moniliformis* were present because the morphology of all *Moniliformis* species is very similar. They tentatively identified the eggs as *Moniliformis clarki* because that species was known to be well represented in the rodents of the area (Fry and Moore 1969). Likewise, a 1974 investigation of the Glen Canyon, Utah, coprolites discovered fluke eggs, but was unable to pinpoint an exact identification of fluke species because the morphology is very similar among the species (Moore, et al. 1974).

These early studies identified a number of other parasites in the America's, primarily in the area of the American Southwest and the Colorado Plateau, where conditions are most conducive to preservation. Additional studies from South America have been conducted. Reinhard, et al. (1985) list eleven intestinal parasites that had been identified in the New World, see Table 2.

Table 2: Intestinal parasites identified in prehistoric humans up to 1985.

Parasites in the New World up to 1985	Usual Hosts	Common Name
Enterobius vermicularis	Human obligate	Pinworm
Ascaris lumbricoides	Human, often co-infected with Trichuris	Roundworm
Trichuris trichiura	Human, often co-infected with Ascaris	Whipworm
Strongyloides spp.	Human, Dog	Threadworm
Trichostrongylus spp.	Primarily herbivores, Human	Hairworm
Ancylostoma duodenale	Human	Hookworm
Moniliformis spp.	Rodents, Dogs, Foxes	Thorny headed worm
Taeniids	Multiple species – generally larger	Tapeworms
Hymenolepids	Multiple species	Tapeworms

Since 1985, a number of other intestinal parasites have been identified, and examination of possible zoonotic parasites (those primarily infecting animals but posing a health risk to humans) have been undertaken (Fugassa, et al. 2011; Jiménez, et al. 2012). New parasites discovered include *Echinostoma spp.* and *Physaloptera spp.* and *Diphyllobothrium spp*. (Cleeland, et al. 2013; Patrucco, et al. 1983; Sianto, et al. 2005).

Coprolites, latrine sediments, burial sediments and intestinal contents are the primary sample types associated with archaeoparasitological work and these studies have been yielded valuable information, even with the inherent limits in relation to morphological identification due to the close similarities between eggs, larvae and worms in many cases. For more information, several

good reviews have been published and the reader is directed to these: (Bryant and Dean 2006; Faulkner and Reinhard 2014; Heizer and Napton 1969; Horne 1985; Kliks 1990).

Sanger sequencing and paleoparasitology

Molecular methods have been widely used to study ancient parasites. The application of molecular techniques to parasitological analysis began in 1999 with the genetic isolation of a ~330bp of DNA of *Trypanosoma cruzi*, the causative agent of Chagas disease, extracted from desiccated organs of mummies from northern Chile dating to about 4,000 years BP (Guhl, et al. 1999). In 2000, Li et al. applied molecular techniques to characterize the fish parasite *Ligula* from formalin-fixed museum specimens. Their study also characterized the connection between formalin as a preservative and its effect on DNA degradation (Huijsmans, et al. 2010; Li, et al. 2000; Murray, et al. 2000). Figure 5 shows a timeline of aDNA noting beginning of aDNA research by noting the first cloning studies, and then focusing on molecular parasitological studies.

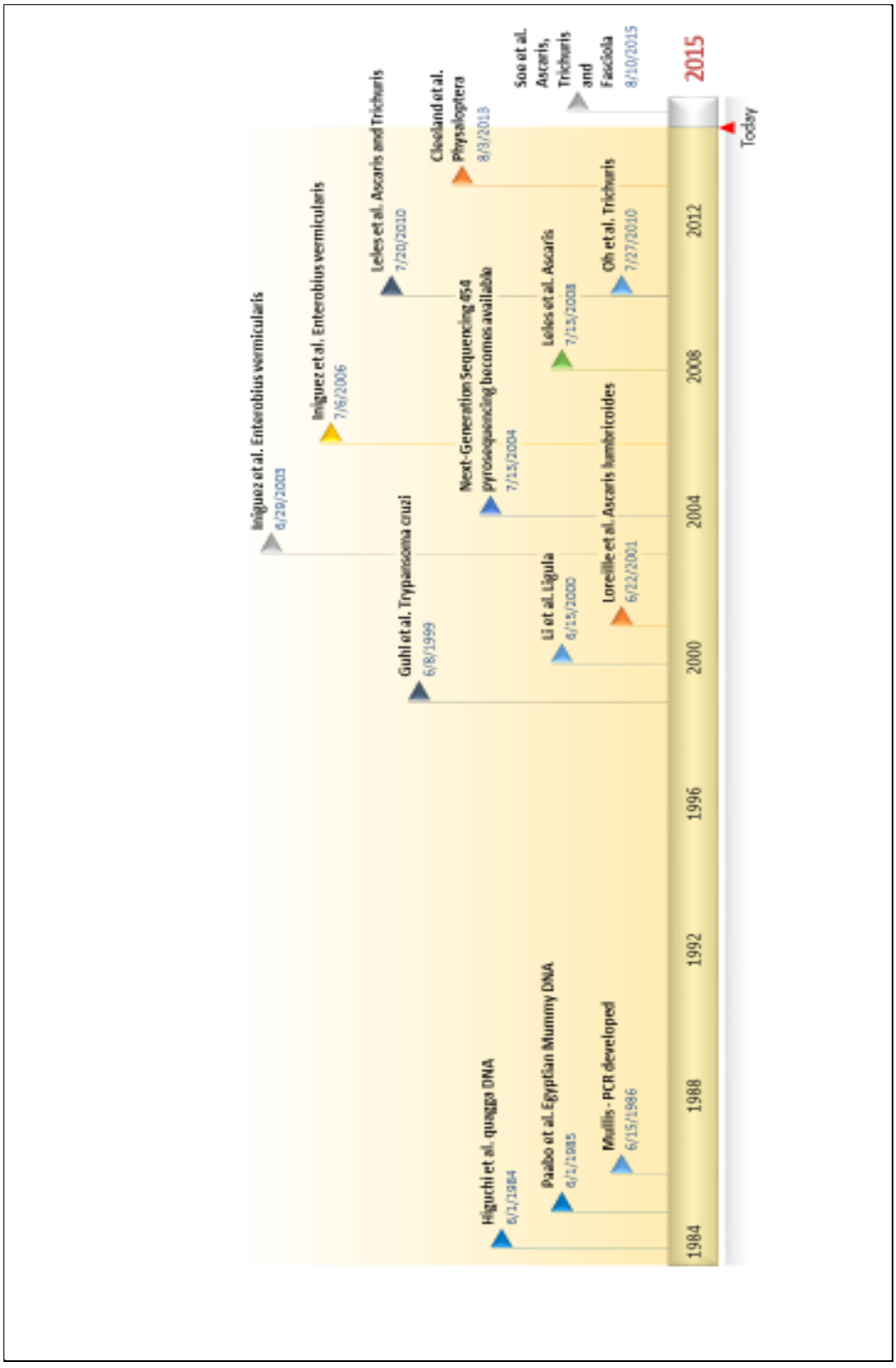

Figure 5: Timeline from the first cloned studies focusing on prehistoric parasite studies.

Loreille, et al. (2001) reported on the successful extraction, amplification and sequencing of *Ascaris* eggs from coprolites from a medieval site in Belgium. This was the first report of the amplification of intestinal helminths from human coprolites. Iñiguez, et al. (2003) published the first molecular paleoparasitological approach identifying *Enterobius vermicularis* in ancient coprolites from North and South America. They followed this study by isolating the SL1 RNA gene from pre-Columbian human coprolites, in order to address whether or not the direct retrieval of sequences from fecal material was possible, without a prior microscopic examination (Iñiguez, et al. 2006). *Enterobius* is the common pinworm, a human obligate parasite that is an ancient nuisance for humans and is easily transmitted by the fecal-oral route or through the air. It is, however, difficult to diagnose *Enterobius* morphologically as relatively few eggs are passed within fecal matter. Iñiguez, et al. (2003) compared the microscopic and molecular capture rates. Molecular analysis confirmed six positive and nine negative microscopic diagnoses. The molecular analysis was unable to confirm nine of the microscopic positives but captured *Enterobius* genetic material in three specimens that were negative by microscopic examination (Iñiguez, et al. 2003). This study demonstrated the potential of using molecular methods to capture evidence of an organism's presence even in the absence of visible remains.

Ascaris is a popularly studied genus. This parasite is exceptionally important today to both human and economic health. *Ascaris* eggs are

extremely durable and can remain viable in soil for up to fifteen years (CDC 2013; Leles, et al. 2008; Loreille, et al. 2001; WHO 2014). More than 1.4 billion humans are affected with *Ascaris* (WHO 2014). Archaeologically, *Ascaris* is ubiquitous and has a deep history with humans. In fact, the earliest known cases of *Ascaris* infection were found in a 30,000-year-old site in France (Loreille and Bouchet 2003).

Evolutionary studies of *Ascaris* have recently been a major topic of parasite research. Classically, there are two known species of *Ascaris*: the human parasite *Ascaris lumbricoides* and the pig parasite *Ascaris suum*. *A. lumbricoides* was identified by Linnaeus in 1758 and *A. suum* identified in 1782 by Goeze (Loreille and Bouchet 2003). There has been extensive discussion regarding which of the species came first, whether the human variant became the porcine variant or vice versa (Leles, et al. 2012). While adults can be distinguished more easily morphologically, the eggs, however, cannot be distinguished and attempts to utilize alternative methods such as immunological and biochemical differentiations have been less than definitive. Loreille and Bouchet (2003) argue for the importance of paleogenetics as a powerful and efficient tool, which can aid in the understanding of *Ascaris* evolution, and they called for the collaboration and multi-disciplinary research approach that involves multiple specialties including archaeologists and parasitologists as well as geneticists. Leles and colleagues provided a comprehensive review of paleoparasitological, genetic and newer evidence of the two *Ascaris* species and argued that they reflect a single species; therefore, *A. suum* should be

synonymous with *A*. *lumbricoides* instead of a distinctive species in its own right (Leles, et al. 2012).

Historically, *Ascaris* and *Trichuris* are often found as a co-infection in individuals. Leles, et al. (2008) undertook a molecular study of *Ascaris* in pre-Columbian South American coprolites to test the association of *Ascaris* and *Trichuris* infections based upon microscopic analysis. In a review of published material, Leles, et al. (2010) notes a paradox in that the New World seems not to show a strong association; they found that 10 of 18 samples for North America had a co-infection of *Ascaris* and *Trichuris*, while in South America only 2 of 19 samples were co-infected. Leles, et al. (2010) suggest a number of possibilities as to why this association might break down in the Americas, including the presence of nematophagus fungi, differential preservation rates, and differential susceptibility to vermicidal substances, which might destroy *Ascaris* at a greater rate than *Trichuris*.

The study of modern parasitism is challenged by the ability to detect parasites that are often in low frequencies. Researchers of modern parasitism argue for a combined microscopic and molecular approach to parasite diagnosis (Bott, et al. 2009), archaeological researchers also argue for a combined methodology (Cleeland, et al. 2013) as does this dissertation. Carlsgart, et al. (2009) developed and tested a method, which was sensitive enough to isolate and amplify DNA from a single unembryonated *Ascaris* egg. successfully isolated DNA from a single helminth. These situations are not unlike the challenges of ancient DNA research. Because ancient DNA is

degraded and present in low copy number, molecular tests are designed to amplify low-copy, short fragment, DNA. In Chapter 3, ancient parasite DNA was extracted and amplified from microscope slides used for morphological analysis, as published by this author (Cleeland, et al. 2013).

Oh, et al. (2010) provided a model example of an ancient DNA parasitological study. The researchers carefully followed ancient DNA criteria of authenticity (Pääbo, et al. 2004). Oh, et al. (2010a) were the first published ancient DNA study of *Trichuris trichiura* (aka whipworm), a common tropical parasite. They designed two sets of overlapping informative primers of less than 200 base pairs (bp) each, which resulted in a combined sequence of ~255 bp of DNA. Each test was replicated in an outside independent laboratory and all results matched. More surprising in their study was the source material; rather than a visible coprolite, the material was from sediments around a skeletal burial, near the lower abdomen. This serves as a proof of concept, that molecular paleoparasitology is not dependent on intact coprolites. Oh, et al. (2010b) also reported on *Ascaris* DNA from an ancient East Asian burial. This same research group has performed a number of paleoparasitological studies utilizing both traditional morphological and molecular methods, including a 2012 study identifying trematode infection in a female mummy. The trematode infecting the individual was confirmed via molecular analysis and was a species of *Paragonimus,* one of the most insidious and prevalent trematodes causing infection world-wide (Shin, et al. 2012).

Coprolites: Next Generation Sequencing

The use of massive parallel, high throughput Next Generation Sequencing (NGS) has not been applied specifically to archaeoparasitological analysis, although this study will cull information from previous NGS runs of ancient material for parasite information.

NGS technology have been applied to ancient samples in two studies using two different methodological approaches. Tito, et al. (2008) used a shotgun direct sequencing method to determine the phylotypes (a taxonomic inventory) and the functional profiles (gene inventory) of the bacterial communities in two coprolites from the El Zape site in Durango, Mexico dating to about 1400 years BP. In 2009, Tito and colleagues utilized a targeted method to isolate the 16s rRNA gene in the bacterial communities in six ancient coprolite samples from three different geographic regions and compared the results to each other and to other published gut biomes from modern individuals as well as Ötzi the Tyrolean Ice Man and a WWI pilot recovered from a glacier 93 years after his disappearance. The results suggested that one of the ancient samples was very similar in bacterial composition to modern children from rural Africa. The study also supported that the composition of the ancient samples were similar to human gut microbiomes, but also the coprolites from the same geographic region were more similar to one another than to modern samples or coprolites from different regions. The authors propose that globalization, industrialization, and modern medicine have significantly altered the human gut microbiome in modern populations (Tito, et al. 2012).

In relation to specific applications to parasites, there have been studies on the malaria agent of *Plasmodium relictum* in the Hawaiian Islands to identify mitochondrial diversity (Jarvi, et al. 2013). Intra-host diversity for the protozoan parasite *Cryptosporidium parvum* has also been characterized by NGS (Grinberg, et al. 2013). A study on *Leishmania donovani* from clinical isolates using NGS discovered co-infections with a related genus of *Leptomonas* in patients in India (Singh, et al. 2013). The Illumina NGS platform was used in a 2011 study to compare *Trichinella murrelli* with the human *Trichinella spiralis* to better understand the divergence between the two and assess whether *T*. *murelli,* most often found in wild hosts, might pose a problem to free ranging livestock and thus present a risk to human health as well (Webb and Rosenthal 2011). *Necator americanus*, the most prevalent hookworm worldwide and the agent of serious health consequences for humans was subjected to 454 NGS in a functional analysis to identify key genes and their products in order to identify potential targets for new drug therapies (Cantacessi, et al. 2010).

While the technology is available to move forward with NGS technology applications, there are still difficulties in adapting the use for ancient samples. Dittmar (2009) argues for the use of NGS technologies for ancient DNA, suggesting a number of ways the technologies and the data they produce can be utilized for archaeoparasitological analysis (Dittmar 2009).

Human Parasitism

Humans have a long evolutionary history with parasitic organisms, which thrive in the larger environmental system and inhabit the human body or

portions of the human body as their specialized niche (Bundy 1988; Fellous and Salvaudon 2008; Hoberg and Brooks 2008; Morgan and Wall 2009; Rosenthal 2008). The association between parasites and humans is sometimes neutral, sometimes beneficial but more often than not it is detrimental to the host (Bogitsh, et al. 2013; Combes 2001; Fellous and Salvaudon 2008; Mbora and McPeek 2009; Santoro, et al. 2003).

Parasitism is a broad term that encompasses any organism which utilizes another for its sustenance and growth (Bogitsh, et al. 2013; Combes 2001). Parasites often but not always co-opt the host's nutrient supply and other physiological functions for its own use, diverting resources from the host to the parasite, which can lead to detrimental effects on the host (Bogitsh, et al. 2013; Combes 2001; Stephenson, et al. 2000b). This paper is specifically concerned with intestinal parasitism of human hosts by helminthic nematodes.

Archaeologically, a rise in parasitism corresponds with the advent of agriculture as viewed through microscopic analysis of coprolite and soil sediments from ancient sites (Reinhard, et al. 1988).

Recently, evidence suggesting that climate change can also lead to increased parasitism, echoes earlier researchers (Morgan and Wall 2009; Penner 1941). Anthropogenic alterations to the landscape, especially in relation to the intensification of agricultural subsistence, could be a main cause of such changes. Such ecological changes result in the unintended consequence of increased parasitism (Cort 1942; Gillespie and Chapman 2008; Matson, et al. 1997; Mbora and McPeek 2009; McCallum and Dobson 2002; Morgan and Wall

2009; Penner 1941; Rosenthal 2008; Wasserberg, et al. 2003). Anthropogenic changes to the environment are not the only human mediated action that affects parasitism, human behaviors from aggregation to length of infant nursing all have some bearing on the intensity and exposure to parasitism (Cort 1942; Larsen 1995; Santoro, et al. 2003).

Directly related to human behavior is the concept of macroparasitism as first defined by William H. McNeill (1998). Macroparasitism is defined as the act of being parasitized by a large-bodied organism, which includes humans. McNeill argued that as humans gained dominance as hunters and became the apex predator it parasitized other animals and other humans as food sources. When agriculture became the dominant lifeway this parasitism was modified to suit the new conditions, resulting in the taking of food resources in a variety of ways from the spoils of conquest, to forced labor and surrender of harvests or through taxation and rents in the form of food supplies provided to humans in power. Intimately tied with social inequality and labor differentiation, these parasitic relationships can become one-sided, with the parasitized group carrying the heaviest burden and paying the heavier costs in regard to diminished health and vulnerability to microparasitic infection (McNeill 1998).

In the case of the inhabitants of Zape, it is possible that some form of macroparasitism is at play. The Loma San Gabriel culture is peripheral to the larger and more complexly organized Chalchihuites cultures and may even represent a peasant elite relationship with the neighboring Chalchuihites as proposed by Hers (1989)

As with all ecological phenomena, parasitism is a complex interaction between environment, host and parasite, interacting on multiple levels and affecting each other in sometimes unforeseen manners (Combes 2001; Gurarie and Seto 2009; Zaccone, et al. 2006). Untangling these interactions, however, is vital to the understanding of the system and where mediation will be most beneficial without causing harm. Recent research into autoimmune diseases in Westernized countries suggest that there is such a thing as too few parasites (Zaccone, et al. 2006). The function of parasites in the development of a properly functioning immune system is little understood, but of vital importance in the eradication of essentially man-made diseases, such as Crohn's Disease of the intestinal tract (Holt 2000; Zaccone, et al. 2006).

For many parasites, interaction begins in the intestine at the mucosal interface, where parasites are recognized and human immune responses are triggered. It is also at this point that the parasite will attempt to evade the host immune defense. If the parasite is successful, the host will become parasitized. However, often in a healthy host, with a properly primed immune system, the parasite is expelled from the body without establishing itself (Bundy 1988; Urban, et al. 1989). Combes (2001:447) warns that underestimating the amount of damage even seemingly innocuous parasites cause must be reversed as new evidence suggests there is always a cost, even if not readily apparent.

The World Health Organization (WHO) estimates that almost two billion people are today infected with one or more of the three main Soil Transmitted Helminths (STH), which are of importance to this study, *Ascaris lumbricoides*

(roundworm) , *Trichuris trichiura* (whip worm) and *Necator americanus* or *Ancylostoma duodenale* (hookworms) (Bogitsh, et al. 2013; CDC 2013; Stephenson, et al. 2000b; WHO 2014). *Strongyloides steracoralis* is the fourth most important STH impacting the health of modern humans (CDC 2013; Stephenson, et al. 2000b). All four parasites can cause severe morbidity and mortality among those infected, disproportionately impacting the health of children, especially infants and toddlers of pre-school age (Stephenson, et al. 2000b). The severity of infections is directly proportional to the parasite load and co-infections (Bogitsh, et al. 2013). *Strongyloides* presents a unique situation among immunocompromised patients today, especially those who have received solid organ transplants. Solid organs are the heart, liver, kidneys, or lungs, as opposed to liquid organs or tissues like bone marrow, skin, or blood vessels. Strongyloidiasis among transplant recipients can be a reactivation of a dormant infection in the recipient or can be donor derived, immunosuppressant drugs increase a condition known as hyperinfection, which increases the parasite burden and accelerates the process of auto-infection, an adaptation of *Strongyloides* which allows it to bypass its normal obligation for time in the soil during its lifecycle (Issa, et al. 2011; MMWR 2013; Roxby, et al. 2009). Another characteristic of hyperinfection is the spread of *Strongyloides* throughout the body to organs it normally does not infest. Mortality in hyperinfection is very often high and swift (Bogitsh, et al. 2013; Chokkalingam-Mani, et al. 2013; Issa, et al. 2011; Kassalik and Mönkemüller 2011; MMWR 2013; Roxby, et al. 2009; Ziad El Masry and O'Donnell 2005).

Children, as noted above, are disproportionately infected with intestinal parasites. Parasitic infections in children have considerable health consequences, representing a significant cause of nutritional and energetic stress (Stephenson, et al. 2000a; Verhagen, et al. 2013). Hookworm infections are associated with anemia. Nutrients can be lost by vomiting, diarrhea, blood loss, blocked absorption or co-optation by the parasite. Nutritional perturbation includes loss of vitamins, minerals, lipids, and amino acids, sugars and proteins. Some of the perturbation is mechanical, *Ascaris* is associated with intestinal blockages and tissue damage and *Trichuris* is a primary cause of rectal prolapse (Bogitsh, et al. 2013: 295, 304-305, 311, 316-318; Kassalik and Mönkemüller 2011; Papier, et al. 2014; Saldiva, et al. 1999; Stephenson, et al. 2000a ; Stephenson, et al. 2000b). Tissue damage is largely caused by larval migrans – parasites migrating through tissues during its lifecycle, for example *Ascaris* spends time in the lungs as do the hookworms and *Strongyloides.* The hookworms and Strongyloides both enter the body by penetrating the skin (Bogitsh, et al. 2013; CDC 2013).

The severity of parasitic infections in children is of exceptional concern to modern clinicians, and may have been a major factor in high infant mortality in prehistoric groups, where the majority of deaths occurred in children five and under and 44% of these deaths occur within the first 28 days of life (Hill, et al. 2012; Unicef, et al. 2014).

Coprolites provide direct evidence of prehistoric parasitism, unlike indirect evidence from soil or latrine samples. Parasites have been identified in

coprolites from prehistorically inhabited dry caves in the Colorado Basin, Arizona, Utah, Kentucky, Durango, Mexico, South America and evidence suggests, subsistence is linked with the incidence of parasitism. For example, the Hinds Cave coprolites from the Lower Pecos region of Texas, are nearly free of parasites, while other groups, such as those at Antelope House, Arizona contain a variety of parasites (Hill, et al. 2012; Reinhard 1988, 2006; Reinhard, et al. 1985; Reinhard and Araújo 2008; Reinhard, et al. 1988; Reinhard, et al. 1987; Unicef, et al. 2014). This suggests that parasitism is variable across time, space and lifeway.

The study of parasitism in prehistoric populations has been used to identify issues related to health (Jiménez, et al. 2012) and migration (Araújo, et al. 2008) and can also be used to infer diet and resource exploitation, as well as trade and social interaction (Vitone, et al. 2004). Sianto, et al. (2005) suggest that one of the most important reasons for investigating prehistoric parasitism is documenting previously unknown or undocumented human parasites for modern consideration. Additionally, techniques developed in studying ancient parasitism are directly applicable to modern parasitological study. This study provides a number of considerations of merit for modern clinicians.

Caveat on coprolite identification of origin

Perhaps the most important preliminary question regarding coprolites, is what produced them? Which creature defecated that particular fecal bolus? The answer to this question, has implication for all other answers obtained. For example, if the coprolite is human, what parasites were causing issue? If the

coprolite is canine, what parasites impacted canine health and which ones could also have caused zoonotic disease for humans? While preservation in caves is generally excellent, caves are not exclusively inhabited by humans, therefore there is always a possibility that the fecal matter belongs to a non-human source.

Of special interest and potentially confounding is the close association of humans and canines. Additionally, the patterns of behavior of both canines and humans can further confound the origin of coprolites. To date there is no definitive test for the original depositor of a coprolite, and therefore, multiple lines of evidence must be evaluated in order to infer the original depositor.

Canines are classified as carnivores but actually are omnivorous. It is not unusual to find grasses, fruits, vegetables and seeds within a canine fecal sample, especially domesticated canines who live in close proximity to humans (Chame 2003). Axelsson, et al. (2013) identified genetic adaptations that increased the ability for dogs to consume diets rich in carbohydrates. Dogs are also coprophagic (Nijsse, et al. 2014; Pinheiro, et al. 2011), meaning they eat feces. Canines are necrophagic, meaning they will eat dead flesh or carrion, including human flesh (Steadman and Worne 2007). Historic references show dogs often fed on corpses and were sometimes given captives to eat (Bressani 1901; Jeune 1901; Lallemant 1901a, 1901b; Steadman and Worne 2007). Bhadra, et al. (2013) in a series of experiments with feral scavenging dogs utilizing human disposal and dump sites, found dogs had a preference for items that smelled like meat and would gobble those items first whether they were

true meat or only smelled of meat, but they would not forgo carbohydrates in food seeking. They argue this plasticity allows for efficient scavenging. Dogs historically have scavenged human waste areas. They have also been treated in differing ways by Native groups across the Americas. Historical references document both the refusal to feed dogs as well as feeding dogs from their own table and scraps from the tables (Charlevoix 1761; Lallemant 1901a, 1901b). All of these lines of evidence suggest that human DNA can find its way into canine coprolites, as well as items traditionally associated with human fecal composition can be found in canine coprolites.

Conversely, canine DNA can be found and should be expected in prehistoric contexts due to the historical references to human consumption of dogs (Charlevoix 1761; Jeune 1901; Lallemant 1901a, 1901b; Lambourville 1901; Maximillian 1906). Archaeological evidence of the importance of dogs for human lifeways are abundant. The disarticulated bones of dogs are often found with other food refuse in archaeological contexts. Kerber (1997) provides an excellent summary of both archaeological and ethnohistorical examples of dog treatment as companions, hunters, sacrifices, and food in the eastern parts of North America. Thurman (1988) in seeking the identity of the Chariticas or “dog-eaters” described in multiple accounts, expands the documented practice of dog-eating from the Canadian Arctic area, through the high northern Plains, and into the central Plains, the Great Basin, the Southwest and the southern Plains. Thurman also highlights that the name “dog-eater” is found in both Athabaskan and Algonquin language families. Thurman noted that several groups who ate

dogs, selectively bred and raised dogs for both transport and food. Levanthal, et al. (2012) documents this practice in central California from the Archaic to the Historic period. Burleigh and Brothwell (1978) document the practice of dog-eating and dog breeding into Mexico and down to South America in Peru and Ecuador using isotope analyses. Perhaps, of most importance, for this paper, the Colima dog dating to about 600 AD is represented by ceramic figurines depicting corpulent small hairless dogs bred specifically for food and fattened on corn. This provides at least three accounts of small dogs bred specifically for food. Bay-Petersen (1983) extends the practices of breeding and eating dogs to the Polynesian islands.

The first direct archaeological evidence of dog consumption by humans was the discovery of a canid cranial condyle within an intact human coprolite from Hinds Cave, Texas, a rock shelter site along the Lower Pecos River. Molecular analysis confirmed that the element, a cranial condyle, was indeed dog and not coyote or wolf (Tito, et al. 2011). Because the identification of canid remains: wolf, coyote and dog is problematic morphologically, the use of aDNA is essential to secure identification. Levanthal, et al. (2012) identified a number of canid remains using aDNA analyses from both Archaic dog burials and later Dog Feasting events and found that all of the remains were dog. The finding (discussed in Chapter 4) in this study is the first identification of dog within a human coprolite in the absence of intact physical remains. Further testing is needed to replicate this finding.

There have been no published descriptions of the faunal assemblage of the Zape site, but information relating to the fauna still available in the area and present in the archaeological material indicate that coyotes were hunted by the inhabitants of the Zape site (Brooks, et al. 1962). Targeted PCR for canine primers and a subsequent phylogenetic tree building would confirm that the finding is dog and not coyote and that the skeletal elements related to coyote are in fact dog. Finding the faunal remains from the site may prove problematic, thus testing of the actual skeletal material from the site may not be possible.

Chapter Three: Methodology

This study undertakes two different molecular approaches to examine the potential parasite inclusions in coprolite sample Zape 23 from the *La Cueva de los Muertos Chiquitos*, archaeological site north of the town of El Zape, Durango, Mexico (Brooks, et al. 1962). The coprolite was recovered during the first of two excavations at the site, during the summer of 1957 by Dr. Richard H. Brooks and Dr. Sheilagh Brooks. The coprolite was recovered from unit B4, beneath a cap of puddled adobe floor, in association with both human burials and a habitation midden (Brooks, et al. 1962; Brooks and Brooks 1978). This unit was dated to the earliest occupation based on an associated piece of wood in the midden, dating to about AD 660 (Brooks, et al. 1962). This area represents one of two puddled adobe floors over the midden and associated adobe partitioning of the area. The second floor, also covers burials, which are disarticulated, with the exception of a single *olla* burial of a three year old child. The second floor postdates the first and is representative of a later time period. The second floor covered an area that had been heavily disturbed by rodent activity, while the oldest floor, located closest to the cave wall had only minor rodent activity, impacting only one burial area (Brooks and Brooks 1978).

The study was approached from a complementary and combined methodology, which incorporated traditional morphological parasite identification, with innovations, such as, extracting DNA directly from the microscope slide followed by targeted PCR methods (Cleeland, et al. 2013). As

such, there are some preparatory methods, for aDNA that will also be presented, these include: positive control development and primer design and testing. No longer are molecular methods limited to PCR analysis. The advent of Next-Generation Sequencing (NGS) technology and methods, allows researchers to perform analyses on samples, which will capture reads of DNA sequences for all or a subset of the organisms within a sample. As coprolites are a complex amalgam of bacteria, viruses, parasites, food remains, pollen and host cells, it is perfectly suited for use in NGS analyses. NGS may be performed as Whole Genome Shotgun Sequencing (WGS), which captures the majority of genetic information within a sample library (a prepared sample or pooled set of samples). Targeted approaches can also be used, for example, targeting only specific genes, like the bacterial 16s rRNA gene, in a targeted NGS, the 16s rRNA gene target will be amplified for analysis from the library, from all sources, and other genetic information will be ignored. NGS produces enormous amounts of data, which must be managed with computer supported bioinformatics tools. The beauty of this data, however, is that once it has been generated, the information is available for multiple analyses, for the present and future. Because a targeted NGS attempt was not possible for this study, data generated as WGS data for Zape 23 from earlier studies have been analyzed in an effort to identify parasite sequences (Tito, et al. 2008). Due to the fact that the reads returned are often short, they are not always able to provide a robust identification. Therefore, data needs to be culled, through a series of filters, that will, ideally, provide the most robust and lengthy sequences for genetic

comparison and identification. These methods and tactics will be more thoroughly discussed in the results and discussion chapter.

Preparatory Methods: Positive Controls

Ascaris lumbricoides samples were obtained from the Texas State Department of Health, via cooperation with the Oklahoma Department of Health and provided by Texas parasitologist, Cathy Snider and Oklahoma parasitologist, Michael Lytle.

Parasites were received in individually capped conical tubes, and suspended in a polyvinyl alcohol (PVA) preservative solution. The samples were stored in the 4 degree refrigerator in the modern lab. They were never processed, stored, or in contact with samples from the ancient lab.

Single parasite specimens were removed from the PVA solution and rinsed several times with Sigma DD H_20. Small segments of parasite tissue were cut using a sterile scalpel and approximately 25 mg of tissue were processed using the Mo-Bio Ultra Clean Fecal Extraction Kit, using the manufacturer's protocols. The Fecal Extraction Kit is specifically designed to remove PCR inhibitors often found in fecal matter therefore parasite samples which were retrieved from fecal material were also processed using the Fecal Extraction Kit.

Parasite sample extractions were then processed using a targeted PCR and previously published primers (Loreille, et al. 2001). Consideration was given to primers that targeted sequence segments less than 200 bp, which are more consistent with the length of aDNA fragments.

Appropriately sized and located samples were then prepared for sequencing to confirm that the genetic sequence was indeed that of the parasite, using the Exo-Sap PCR product cleaning protocol. Samples were then submitted for sequencing to the Sanger Sequencing Laboratory at the University of Illinois, Urbana-Champagne. Returned sequences were trimmed in the Sequencher® computer application (Codes 2014), and then submitted to the NCBI Blast database (Altschul, et al. 1990), for confirmation. The modern *Ascaris* samples positively matched and aligned with the reference sequences obtained from the NCBI database. The modern *Ascaris* extraction solution was marked and placed in a box reserved for confirmed positive samples and stored in the modern lab 4 degree refrigerator.

Alternate Positive Control Samples

While the modern *Ascaris* sample was suitable for use with suspected archaeological *Ascaris* samples and using *Ascaris* primers, it was not necessarily suitable for use as a control with other primer sets or samples. Therefore an alternative development of a positive control, requires using previously amplified and confirmed samples. The use of alternative positive controls were necessary in this study. This was accomplished by reserving the amplified and confirmed via sequencing, PCR products. As new primers were designed and tested, additional confirmed PCR products were retained as positive controls. The disadvantage of using this type of positive control, is that it can only be used in amplifications that use the same primers. Extra care must also be taken with PCR product as a positive control, as they can easily

contaminate archaeological samples not yet processed. PCR derived positive controls are kept in separate boxes in a separate place in the lab refrigerator and are added to the positive control tubes in a dedicated area, to prevent cross contamination of the lab environment.

Primer Choice and Design

For the initial parts of this study, previously published primer sets were used. Described later in this chapter is the design of primers specifically for the organism *Physaloptera*, using the 18s rRNA gene sequence, in an effort to increase the length of the sequence available for a more robust identification.

Sample Origin

The context within which the coprolite was recovered is examined to determine its impact on the origin of the coprolite. The coprolite itself is examined for general shape, size and visible inclusions, such as charcoal, food remains, plant material, hair, bone, seeds, etc. The color of rehydration solutions are noted and the presence or absence of odor after rehydration is also noted. Previous morphological analyses were reviewed for the researchers' opinions on the origin of the coprolite. Of particular interest are the parasites identified via morphological analysis in determining the origin of the coprolite. Previous molecular work was, likewise, reviewed in order to determine if any molecular work performed assessed the potential origin of the coprolite. A discussion of the multiple lines of evidence and the final determination of the origin of the coprolite is supplied in the results and discussion chapter.

Sample Preparation

Initially, two putative human coprolite samples from the Zape site were prepared for molecular analysis, Zape 23 and Zape 29. In the dedicated ancient DNA laboratory, each fecal bolus was removed from the original packaging in which they had been curated. Using a sterile scalpel for each sample, 1 gram of fecal bolus was removed. Because some parasites, such as *Enterobius vermicularis* are more likely to be found on the exterior of the fecal bolus than within its matrix, because of the egg laying habits of the females, there was no attempt to remove the exterior layer of the fecal bolus nor treat it with bleach. In order to prevent these samples from being used for non-parasite analysis ,because of the intact fecal layer they were marked and boxed in a separate container marked, “parasite only” (Cleeland, et al. 2013). Figure 6 is a flow chart of the process from archaeological sample prep to final analyses.

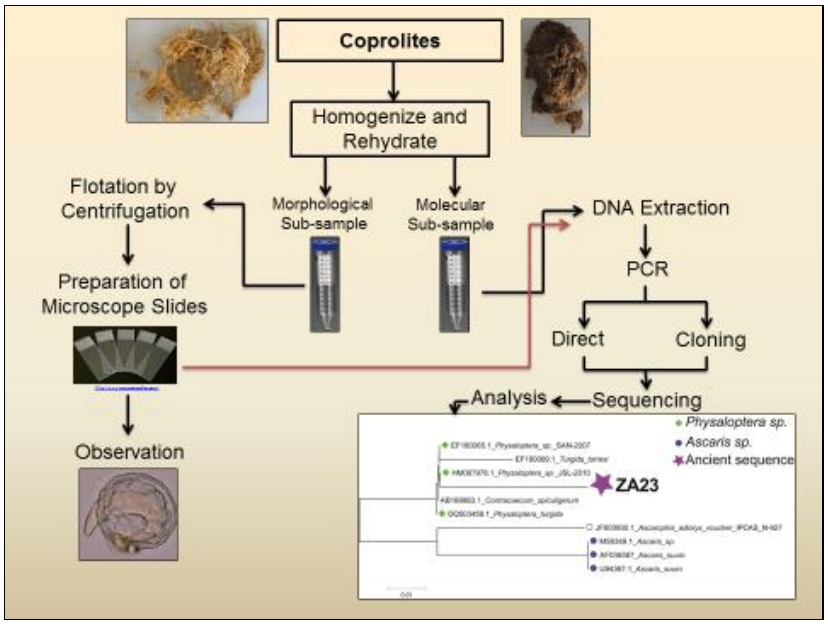

Figure 6: Methodological steps from coprolite preparation to final analysis, using a combined morphological and molecular approach as designed in this study.

Rehydration

The methodology used in this study can also be found in Cleeland, et al. (2013). Each 1 gram subsample of the fecal bolus was transferred to a 15 ml conical tube and 1 to 5 ml of Tris-EDTA pH 8.0 solution was added, depending upon the absorbency of the fecal material. Using the sterile scalpels, the fecal bolus was disaggregated. The tubes were sealed and wrapped with Parafilm®, then secured to an orbiter, for 72 hours to continue the disaggregation and homogenization of the sample. Periodically, during those three days, the tubes would be removed from the orbiter and vortexed, then replaced on the orbiter. After 72 hours, the tubes were removed from the orbiter, vortexed a final time and aliquots of 500µl containing both solution and sediment were prepared for transport to the Veterinary Pathoparasitology Lab at Oklahoma State University in Stillwater, Oklahoma. The 2ml tubes, were sealed, wrapped in Parafilm® and double bagged, before removing them from the ancient DNA lab. Aliquots of sample removed were *never* returned to the ancient lab.

Morphological Analysis

The samples were opened in the Veterinary Pathoparasitology Laboratory at Oklahoma State University in Stillwater, Oklahoma, and transferred to 15 ml conical tubes. Pre-made Sheather's Sugar Solution (water, sugar and formaldehyde) of 1.27 specific gravity was added to the conical tubes to create a reverse meniscus and the tubes were placed into vertical centrifuge that holds the tubes at a 90 degree angle. A microscope slide cover slip was

placed directly on top of the meniscus and the samples were centrifuged for five minutes at 2500 rpms (see Figure 7). At the end of centrifugation, the cover slip was lifted at a 90 degree angle directly up and immediately placed on a microscope slide.

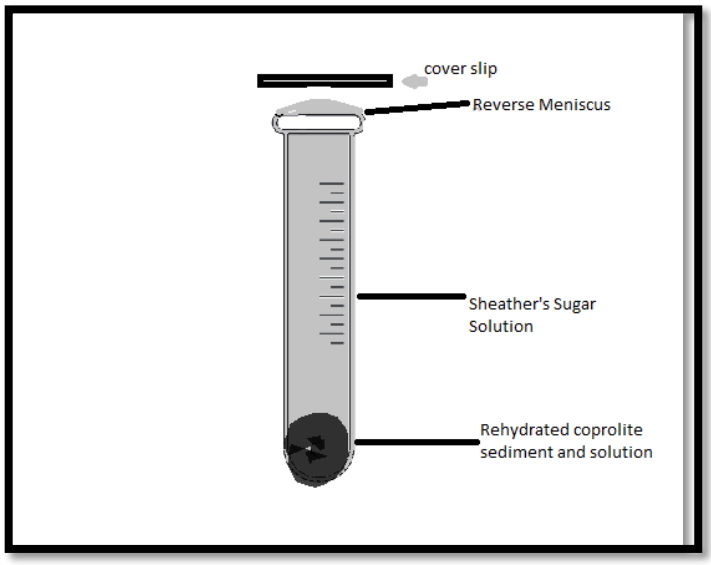

Figure 7: Sheather's Flotation Preparation

The microscope slides were transferred to a compound microscope and scanned at both 100x and 400x magnification. Notes were made of the materials and potential parasite eggs observed. Photographs were taken for Zape 29 only.

The microscope slides were placed in a carrier and returned to the Modern DNA laboratory at the University of Oklahoma.

Microscope Slide Extraction

Based on previous successful extractions made by this author using slides of baboon fecal flotations containing *Trichuris trichiura* eggs, processed using Sheather's Sugar Solution in the Oklahoma State Veterinary

Pathoparasitology Laboratory, the microscope slides for Zape 23 and Zape 29 were opened inside a PCR hood. The slide and the coverslips were carefully rinsed and swabbed, independently, using Sigma DD H_2O. This rinsed solution and the swab tip, were then placed into a 2ml PCR tube and processed using the Mo-Bio Ultra Clean® Fecal Extraction Kit, per the manufacturer's protocol.

Ancient DNA Extraction

From subsamples of the rehydration solution which had been stored in the dedicated ancient lab, in the -20º Celsius (C) freezer, 25µl were extracted using the same kit and protocol as above, with the additional modifications of a freeze and thaw sequence to help crack suspected *Ascaris* eggs in the samples, based upon the morphological analysis. After the sample was added to the bead beating tubes, they were subjected to the following thermal cycle: heated for five minutes at 65º C, freeze five minutes in the -20º C freezer and a final heating of five minutes at 65º C. The sample was then processed using the protocol as per the manufacturer's instructions.

PCR Amplification

Two different targeted PCR amplifications were used in this study. The first, used two different primer sets previously published by (Loreille, et al. 2001) for *Ascaris* 18S rRNA and the second targeted were designed to overlap and be specific for *Physaloptera.* Tables 3 and Table 4 provide the primer set information for the *Ascaris* primers and their PCR thermocycler parameters. Tables 5 and 6 provide the same information for the *Physaloptera* primer set.

Table 3: Previously published *Ascaris* primer sets

Primer sets 18s rRNA *Ascaris*	Primer Sequence	Size	Initial or Additional PCR	Annealing Temperature	Citation
Asc 6 Asc 7	cgaacggctcattacaacag tctaatagatgcgctcgtc	~123 bp	Initial	52º C	Loreille et al. 2001
Asc 8 Asc 9	atacactgcaccaaagctccg gctatagttattcagagtcacc	~99 bp	Initial	52º C	Loreille et al. 2001

Table 4: PCR Thermocycler Parameters for *Ascaris* Primers

Steps	Initial Denaturing	Denaturing	Annealing	Extension	Final Extension
Number of cycles	1	60	60	60	1
Temperature	94º C	94ºC	52º C	72º C	72º C
Time Length	2 minutes	15 seconds	15 seconds	15 seconds	5 minutes

Table 5: Physaloptera primer set designed for this study.

Primer set 18s rRNA *Physaloptera*	Primer Sequence	Size	Initial or Additional	Annealing Temp	Citation
Physa18s243Forward Physa18s343Reverse	tgaatagctctggctgatcg caaccatggtaggcacataaac	~100bp	Additional	58º C	Cleeland et al. 2013

Table 6: PCR Thermocycler Parameters for Physaloptera primers.

Steps	Initial Denaturing	Denaturing	Annealing	Extension	Final Extension
Number of Cycles	1	60	60	60	1
Temperature	94º C	94º C	58º C	72º C	72º C
Time	2 minutes	15 seconds	15 seconds	15 seconds	5 minutes

With the exception of the PCR set-up for the microscope slide extraction, all work was carried out in the dedicated Ancient DNA laboratory at the University of Oklahoma and all aDNA protocols were followed. All work was performed in an enclosed UV irradiated and bleach cleaned PCR hood. The lab itself is a positive pressure clean room with a positive pressure class 10,000 HEPA filtered ventilation system and total lab UV irradiation capability. Researches wear full Tyvek suits with hoods, masks, safety glasses and two pairs of gloves, at all times in the ancient lab. The PCR hoods are bleach cleaned before and after work sessions and are UV irradiated before and after the work session using their embedded UV light system. The lab itself is also UV irradiated for three hours between work sessions.

Using the Platinum Taq Amplification System (Ivitrogen 10966-018), 30 µl reactions were made using the following concentrations: 3µl 10X buffer, 0.9 µl 10mM dNTPs, 1.5µl 50mM $MgCl^2$, 1.8µl of EACH 5µM primer. 0.1µl of 5U/µl Platinum Taq Polymerase, 16.9µl of Sigma ddH^2O and 4µl of 10ng/µl DNA template from extraction solutions. Included with the samples, were an

extraction blank and three PCR controls, in which water was substituted for DNA template, and one tube reserved for a positive control. This protocol uses an increased amount of $MgCl^2$, based upon the protocol of Loreille, et al. (2001). Reactions were created in individually capped PCR tubes and sealed in the ancient lab, prior to being transported to the thermocyclers in the modern lab space. Once in the modern lab, the positive control tube was opened and positive DNA template was added (see Figure 8), the cap was resealed and the tubes placed in the thermocycler under the conditions in either Table 2 or 4.

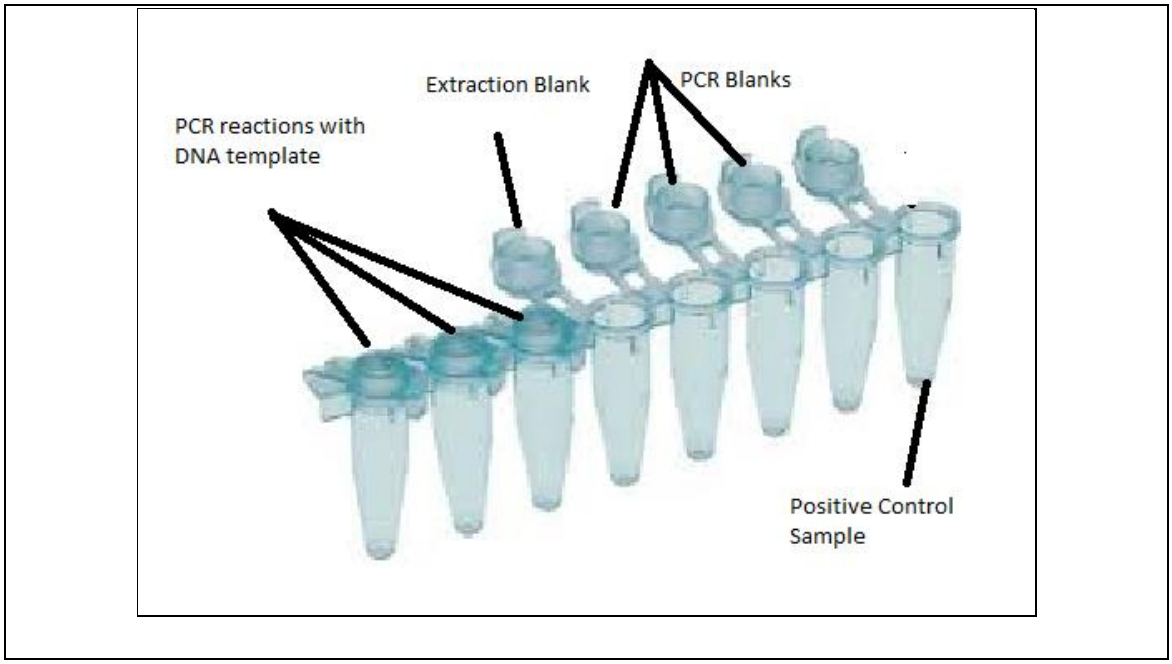

Figure 8: Order of PCR samples and blanks. All eight tubes are sealed in the ancient lab. Tube eight is opened in the modern lab to add positive control DNA template.

In order to visualize and assess the success of the PCR amplification, 8µl of amplified solution were mixed with 2µl of blue 6X loading dye and placed in a 2% agarose gel. The gel was then run at 150 volts for 50 minutes and then placed in an ethidium bromide bath for thirty minutes, and was visualized under

UV light, in the enclosed visualization system. Positive samples were prepped for sequencing using the EXO-SAP protocol and 2 µl were placed in individual wells of a 96 well plate, to which 1µl of 5µM sequencing primers were added. The plates were sealed, packed and shipped to the Sanger Sequencing Laboratory at University of Illinois Urbana-Champagne.

Sequencing

Returned sequence data was uploaded to the Sequencher® software system, trimmed of noise and aligned with the primer sequences. Primer sequences and all data outside of the primer brackets were discarded. The trimmed data were then uploaded to the NCBI nucleotide BLAST program online for taxonomic identification, using a cut-off of 95% identity.

Cloning

PCR reactions were cloned using the TOPO TA® (Life Technologies Catalog # K4530) cloning system and cultured on imMedia™ Kan Blue (Sigma-Aldrich Catalog #28236). Thirteen clones, for Zape 23 were chosen for processing. Clones for Zape 29 failed to replicate. The clones were collected using a pipette tip and were diluted individually in 100µl of ddH^2O. PCR reactions were set up using the M13 universal primers and amplicons sent for sequencing after EXO-SAP cleaning. All steps were performed according to manufacturer protocols. Returned sequences were trimmed and uploaded to the NCBI nucleotide BLAST program online for taxonomic identification, using a cutoff of 95% identity.

Phylogenetic Tree Building

In order to visualize the taxonomic assignment, a phylogenetic tree was constructed using the Neighbor-Joining Method in the MEGA5 program. Reference sequences were drawn from all available *Physaloptera* sequences in the NCBI database for the 18s rRNA gene, as well as 18s rRNA reference sequences for *Ascaris suum*, *Ascaris lumbricoides* and *Contracecum* species. These were pooled and aligned. The Zape 23 sequence was added to the alignment and the pooled samples submitted to a 1000 bootstrap reiteration

NGS – Whole Genome Shotgun Data Analysis

Whole Genome Shotgun sequence read data was analyzed for potential parasite sequences, using data sets previously generated by Tito, et al. (2008). Library construction and NGS protocols can be found in Tito, et al. (2008). The datasets are labeled Zape 23_WG_1 and Zape 23_WG_2. Both used subsamples of the Zape 23 coprolite, but represent two distinct NGS Whole Genome runs and the resulting datasets. The datasets include all of the reads, short segments of genetic sequence captured during the NGS runs. This part of the analysis is bioinformatical in nature. The complete data sets were retrieved from the MG-Rast server (Meyer, et al. 2008), where they have been privately curated since they were originally uploaded to the MG-Rast pipeline for phylogenetic and functional analyses of the bacterial composition of the Zape 23 samples. Table 7 provides and overview of the two 454 pyrosequencing datasets and their compositional breakdown.

Table 7: 454 pyrosequencing data sets as originally processed in the MG-Rast pipeline. Hits suggest sequence matches.

NGS Sample/Datasets Codes	Zape 23_WG_1	Zape 23_WG_2
Date uploaded to MG-Rast	9/02/2008	9/12/2008
Total sequences uploaded to MG-Rast	20,275 sequences	19,091sequences
Total base pairs uploaded to MG-Rast	2,352,585 bp	2,311,045 bp
Average Sequence Length	116 bp	121 bp
Failed Quality Control Standards for MG-Rast	2,690 sequences	2,940 sequences
Bacteria	98.7% (10,846 sequences)	98.6% (9,310 sequences)
Eukaryota	0.7 % (81 sequences)	0.6% (59 sequences)
Archaea	0.3 % (38 sequences)	0.5% (43 sequences)
Other	0.3% (38 sequences)	0.2% (18 sequences)
Chordata Hits	12 hits	18 hits
Nematoda Hits	0 hits	0 hits

Because the majority of each sample represent bacterial information, of each sample, 98.7% and 98.6% respectively were bacterial sequences, it is more difficult to capture the less abundant genetic components. Of the remaining 1.3 and 1.4% of sequences, Eukaryota were 0.7% and 0.6%, representing a total of 140 sequences. This breakdown of the components of the coprolites microbiome is consistent with other findings, which suggests only 1-2% of the biome is other than bacterial in nature (Carpenter, et al. 2013).

In order to enhance the chance of recovering parasite information from this data, the entire raw data set was uploaded and rerun through the Nembase4 database (Elsworth, et al. 2011), which specifically houses Nematode Expressed Sequence Tag data, using the megablast function, which looks for highly similar sequences all data meeting a minimum E-value of 1e- 05

were retained. This resulted in 114 sequences for Zape23_WG_1 and 93 sequences for Zape23_WG_2. These represent our potential parasite data set. However, in order to ensure the most robust classifications, these sequence reads were run through a series of filters, (Figure 9) in order to isolate the sequences with the highest probability of representing authentic and informative parasite sequence data.

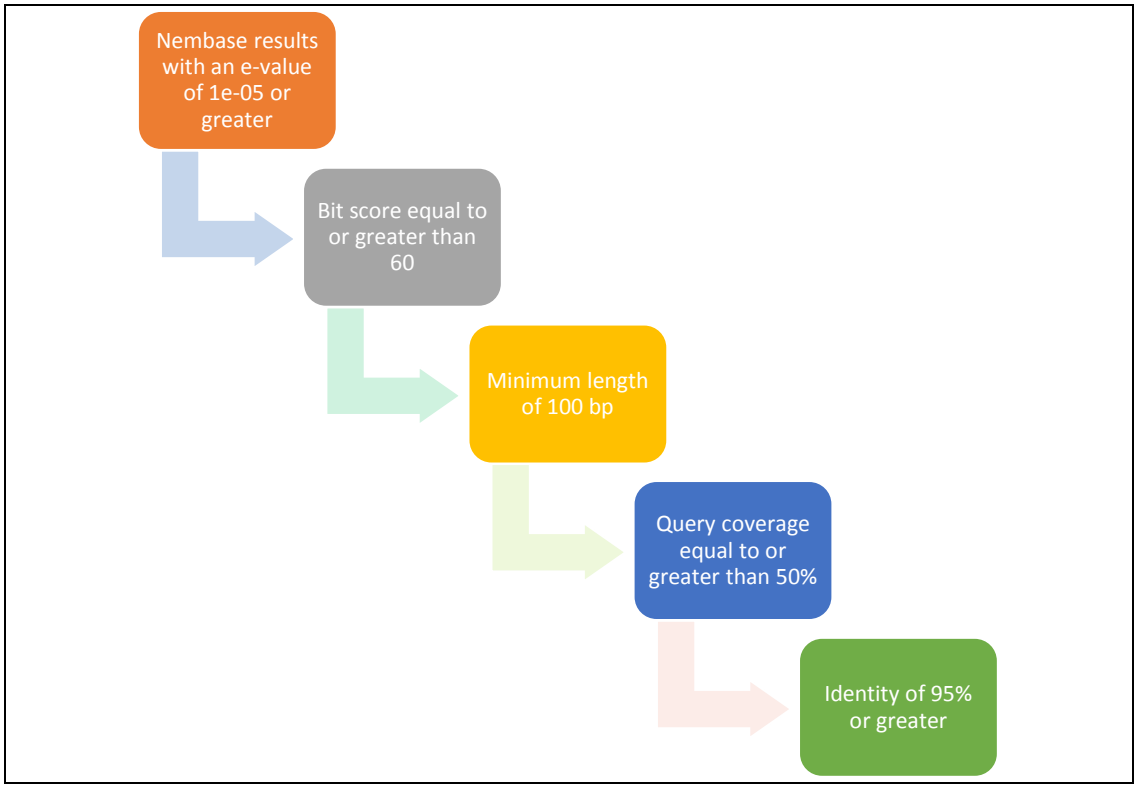

Figure 9: Dataset Filtering Parameters

Table 8 shows the results of each filtering step. The final data set was composed of nine sequences, five sequences from Zape23_WG_1 and four from Zape23_WG_2.

Table 8: Filtering steps and retained sequences.

	Zape23_WG_1	Zape23_WG_2
Total Sequences uploaded to Nembase4	20,275	19,091
Original data set for nematodes	114 sequences	93 sequences
Bit score equal to or greater than 60bits	113 sequences	90 sequences
Alignment length 100 bp or greater	53 sequences	54 sequences
Query Coverage equal to or greater than 50%	53 sequences	47 sequences
Identity equal to or greater than 95%	5 sequences	4 sequences

The final data were then submitted to a number of databases in order to obtain confirmation of the taxonomic assignment. Figure 10 shows the submission process to the various databases, housed on the NCBI site (Altschul, et al. 1990).

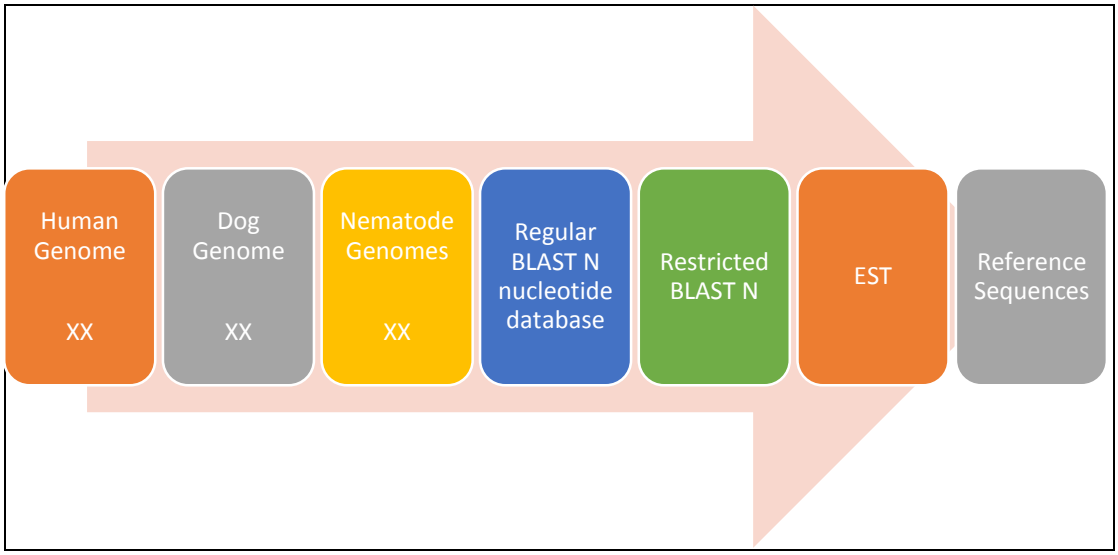

Figure 10: Series of search parameters and NCBI database options used in identifying potential parasite sequences. XX = No information returned.

Each database option or differing parameter, provides a slightly different way of screening the sequences against known and curated sequence information, in order to find the most robust taxonomic assignment (see Table 9 for descriptions of search options employed). The original dataset used the Nembase4 database, which specifically searches for nematode expressed sequence tags (EST). ESTs are unique, short sequence segments from the coding region of expressed (active) genes. ESTs provide landmark and are uniquely informative enough to identify species. All results were then compared for query coverage (the number of nucleotides from the unidentified sequence that matches the nucleotides in a known sequence) and identity (given as a percentage, this suggests taxonomic identity of the queried sequence), with the original Nembase4 assignments, in order to either confirm or reclassify the taxonomic identification.

Table 9: NCBI database descriptions

Database	Description
Human Genome	Compares the query sequence with 455 sequences of human genomic data
Dog Genome	Compares the query against the Can.Fam 3.1 annotated genome sequences totaling 3,268 sequences for dogs.
Nematode Genomes	A restricted Nucleotide database search that compares the query against 6,321 nematode assigned sequences
Regular Blastn	Searches against the Nucleotide database with a nucleotide to nucleotide alignment between query and references. The program can be forced to search the full length of the query. The regular parameters use the megablast parameter to search for highly similar sequences. The regular parameters also include uncultured environmental samples and model organisms.
Restricted Blastn	Is a user modified Blastn search. In this case all bacteria, model organisms and uncultured environmental samples were excluded, all other parameters were not changed. This is to try and remove the bias toward bacterial sequences in the database.
EST	Searches nucleotide by nucleotide through three databases GenBank, EMBL and DDBJ from the EST (Expressed Sequence Tags) division. EST are short unique sequences segments from within the coding region of expressed genes and are used to identify genes as well as landmarks for mapping. They are considered an alternative to organism identification.
Ref_Seq	Compares nucleotide to nucleotide against a database of non-redundant annotated sequences include genomic, transcripts and proteins. It is divided into genomic and rRNA. Selected copies cover all organisms and new genomic references are added as they become available.

Chapter Four: Results and Discussion

Two coprolite samples, Zape 23 and Zape 29 were explored in a number of different manners during the course of this study. Zape 23 was consistently robust and informative, while Zape 29 was less suited to molecular analyses. This is not uncommon despite the fact that both specimens were recovered from the same unit B4, beneath the protective puddled adobe floor, preservation is never the same for all samples. Even the same sample can show differential preservation. Morphologically, however, Zape 29 provided the emerging larva presented at the beginning of this dissertation in Figure 1. Zape 29 provided weak but positive results for *Physaloptera* for *Ascaris* primers 6 and 7 from the microscope slide extraction and the first round of PCR on a second extraction from the reserved rehydrated coprolite subsample. It provided no usable clones during the cloning process, nor did it provide strong results in subsequent PCR, therefore it has been discarded from the majority of the discussion.

The emerging larva from Zape 29, other *ascaris*-like eggs in both samples led us to perform PCR amplification using published *Ascaris* primers as discussed in the methodology. The results however were not *Ascaris* as expected but the rare human parasite of *Physaloptera.* This is the first molecularly confirmed presence of *Physaloptera* in a prehistoric human. Both are nematodes and are cousins on the phylogentic tree, but they are distinct genera and species.

The results will more fully discuss the parasite and its implication for human health. As a relatively young branch of ancient DNA analysis, molecular archaeoparasitology requires specialized methodologies and optimizations. This chapter presents the results of some innovative techniques and discusses some of the areas in need of greater work to bring this discipline to its maximum potential.

Discarding Zape 29

As mentioned above, Zape 23 and Zape 29 were processed in parallel, but Zape 29 failed to provide consistent or robust results. Zape 29 was dropped from further discussion after morphological analysis and the initial PCR run and cloning process because it provided no information.

Identification of Coprolite Origin

Identifying the defecator of a coprolite is essential for downstream interpretation of results. Animals, such as canids, can produce coprolites very similar in form and content to human coprolites. This factor continues to be of interest to coprolite science. The fact that canids are also close companion animals to humans both in the modern world and in the prehistoric world, this can be potentially confounding. Zape 23 was approached as a putative human coprolite, but some questions as to a possible canid origin remain. In an effort to provide some surety to the human identification, multiple lines of evidence have been examined and a conclusion drawn. As noted in Chapter Two, the close association of humans and dogs, plus the behaviors of both, can further confound identification. Data from molecular work, archaeological context,

parasitological work and coprolite analysis are all used to try and determine the origin of this coprolite.

Zape 23 was identified using molecular tools to Native American Haplogroup B (Cleeland, et al. 2013; Tito, et al. 2012; Tito, et al. 2008). Microbiome work on the Zape sample, also indicate similarity to the human gut biome in composition specifically that of a child, as it is similar to the biomes of African children (Tito, et al. 2012).

Archaeologically, the sample was recovered from a habitation site, a culturally modified rock shelter and found within a midden, capped and preserved beneath a puddled adobe floor. This floor was mostly intact. There was no evidence of rodent disturbance in Unit B4, where the coprolites were recovered. A number of sub-adult burials were also in association with the midden (Brooks, et al. 1962; Brooks and Brooks 1978).

The size and cylindrical shape of the coprolite are consistent with human. However, canid coprolites can also be of similar shape. Generally, canid coprolites are encased in a mucosal sheath, excreted by the canine rectum upon defecation (Chame 2003). The rehydrated color was dark black, associated with human feces by some researchers, but additional tests have indicated that the color is more directly related to foods ingested, than the species of the defecator (Bryant and Dean 2006). No odor was detected from the reconstituted sample, as is sometimes smelled with rehydrated samples. Inclusions observed included small bits of charcoal, grass, and plant remains (Bryant and Dean 2006).

Parasites can be indicative of the host species and provide some insight into the defecator. However, this requires host-specific parasites. In this case, the parasites identified in this coprolite are *Dipylidium caninum* by morphology (Jiménez, et al. 2012) and the *Physaloptera* (Cleeland, et al. 2013), both of which are capable of infecting and causing disease in humans as well as canines.

The NGS data which will be discussed later in this chapter, produced at least one read that was a 100% match to *Canis familiaris*, this could suggest that the coprolite belongs to a dog, or it could be molecular evidence of human consumption of dog. Remains found in the cave site, suggested the hunting of coyote (Brooks, et al. 1962). However, morphological differentiation between coyotes and dogs is highly problematic and requires the use of a genetic test to determine the species (Byrd, et al. 2013).

The multiple lines of evidence presented here, suggest that with the given information available, the Zape 23 coprolite is probably human in origin, but it is not possible to definitively rule out a canid origin.

Morphological Analysis

Results of the morphological analysis for the two samples, Zape 23 and Zape 29 were positive, despite the small amount of material processed. Zape 23 produced taenid (tapeworm like) and ascarid (roundworm like) like eggs, as well as plant material, an unidentified parasite body and possible seeds or spores and an unidentified hair. No photographs were taken of the taenid and ascarid like eggs. Zape 29 produced the emerging larva as seen in Figure 1

and possible taenid eggs. Hair, seed pods, an unknown parasite egg and fungal spores were also identified in Zape 29. Based upon the emerging larvae and additional ascarid like eggs, it was decided to pursue *Ascaris* and two previously published primer sets were chosen for use in the PCR analysis.

Because a very small aliquot of rehydrated sediment and solution were submitted to morphological analysis, it was questionable, as to whether or not the step would provide information. However it did provide information and supports the premise that this can be a flexible step, using small amounts or larger amounts of material to process the samples at the researcher's discretion. This step also acts to guide the molecular work to follow, as it provides a starting point for the molecular approach, which decreases the cost of attempting multiple blind PCR analyses.

PCR Amplification – Microscope slide

Extracting the material on the microscope slide and then running PCR on that extraction, is perhaps one of the most important innovations of this study. While there is evidence for the successful recovery of DNA from a single worm, or a single egg, this is not a routine protocol (Carlsgart, et al. 2009; Shayan, et al. 2007). The use of material identified under microscopy, also, provides an excellent and robust method of identifying unknown parasites (or other material), or differentiating between closely related species whose morphology is highly similar and difficult to differentiate. Additionally, this provides a control sample, by which to assess, later extractions and PCRs from the retained rehydrated samples in the ancient lab. It can help assess, whether the finding is

genuine or might represent contamination introduced during the morphological analysis. Ancient DNA is present in small and fragmented copies of a degraded nature, as a result extra care must be taken to ensure that no contamination from robust modern DNA contaminates that sample. The best way to ensure this is by strict in-field acquisition protocols by the archaeologists. To date a majority of aDNA samples have come to the lab after being handled by multiple individuals, who are constantly shedding DNA into the atmosphere. Efforts in the lab to minimize modern contamination are successful in many cases but are not always able to decontaminate all samples sufficiently. This can result in modern DNA results as opposed to authentic aDNA, hence the need for strict aDNA Criteria of Authenticity as discussed in Chapter 2.

When DNA sequence data is returned to the researcher, it is a much longer string of data than the targeted area, this is the result of the primers used and the sequencing protocol, therefore data must be trimmed to remove the primers and the noise produced outside the target region. As primers act as bookend, the sequence of interest is that series of nucleotides found within the bookended primers. The returned sequence data was trimmed and primers removed prior to inputting the samples in NCBI nucleotide Blast program (Altschul, et al. 1990). The sequence results provided 100 percent identity and 100 percent matches to *Physaloptera*. PCR product was then cloned and thirteen clones sent for sequencing. These were processed in the same way, removing the M13 primers as well as the *Ascaris* primers before submitting the sequences to the BLAST program. M13 primers are specifically designed

unverisal primers used in cloning to ensure the capture of target DNA. Twelve of the thirteen clones returned 100 percent matches and 100 percent identity to *Physaloptera* (Cleeland, et al. 2013). The thirteenth clone listed *Contracaecum spiculigerum* as its first best hit. As discussed later, this is a misidentified sequence, therefore all thirteen clones matched *Physaloptera.*

The sequence was identified with 100% coverage and 100% identity to the following organisms: *Physaloptera* sp. SAN-2007; *Contracaecum spiculigerum* (an ascarid) and *Physaloptera turgida.* However, the sequence only returned an 82% coverage and 94% match to *Ascaris suum* and an 87% coverage with 92% match to *Ascaris lumbricoides*, contradictory to the expectations based on the morphological results. (See Table 10). As shown in Table 10 *Physaloptera* breaks away from both *Contracaecum* and *Ascaris* at the Order level and Contracaecum splits from *Ascaris* at the Family level.

Table 10: Taxonomy Comparison of *Physaloptera* spp., *Ascaris* spp., and *Contracaecum* spp.

	Physaloptera	Ascaris	Contracaecum
Super Kingdom	Eukaryota	Eukaryota	Eukaryota
Kingdom	Metazoa	Metazoa	Metazoa
Phylum	Nematoda	Nematoda	Nematoda
Class	Chromodorea	Chromodorea	Chromodorea
Order	Spirurida	Ascaridida	Ascaridida
Super Family	Physalopteroidea	Ascaridoidea	Ascaridoidea
Family	Physalopteridea	Ascarididae	Anisakidae
Genus	Physaloptera	Ascaris	Contracaecum

It is also possible that the organisms were misidentified prior to being sequenced and incorrectly uploaded. Morphological analysis is often hampered by close similarities between species from the level of egg to the adult

organisms. Figure 11 shows the caudal ends of *Ascaris*, *Physaloptera* and *Contracaecum.* Figures 12-14 compare *Ascaris* and *Physaloptera* stages.

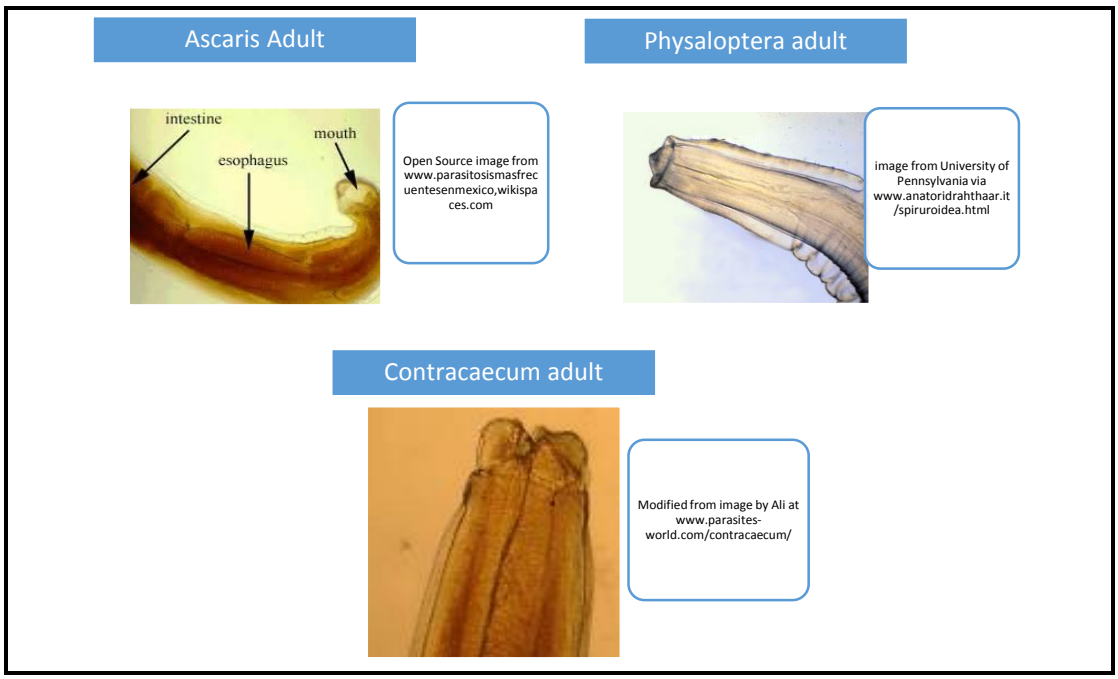

Figure 11: Caudal ends of adult nematodes *Ascaris*, *Physaloptera* and *Contracaecum*

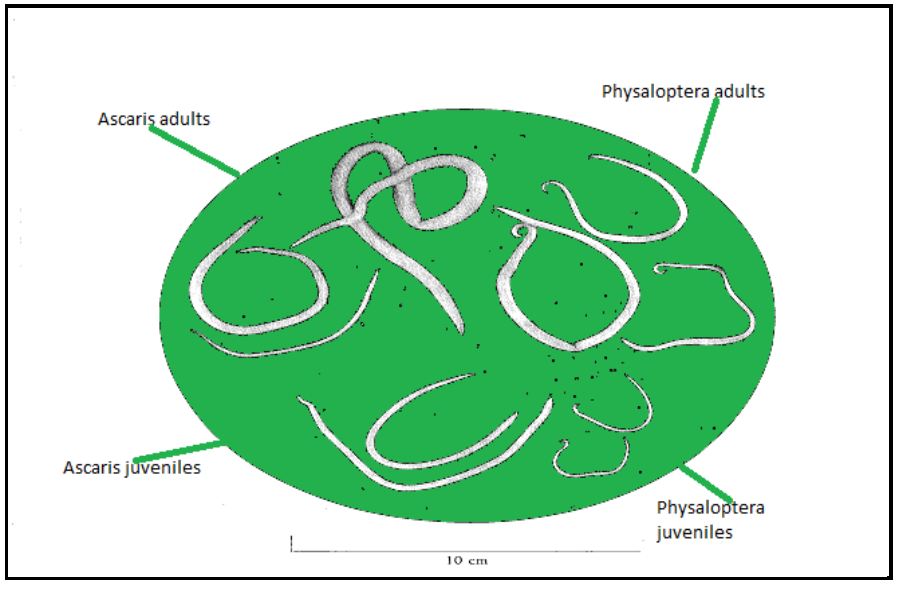

Figure 12: Comparison of *Ascaris* and *Physaloptera* adult and juvenile stages modified from Vandepitte et al. (1964) p. 1072

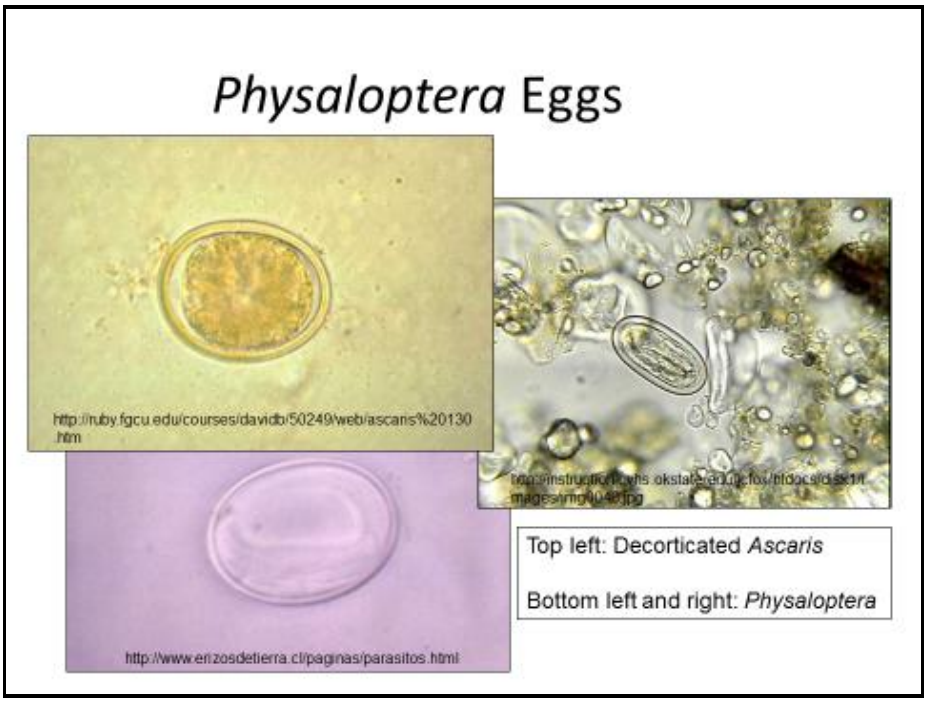

Figure 13: Comparison between *Ascaris* egg and *Physaloptera* eggs

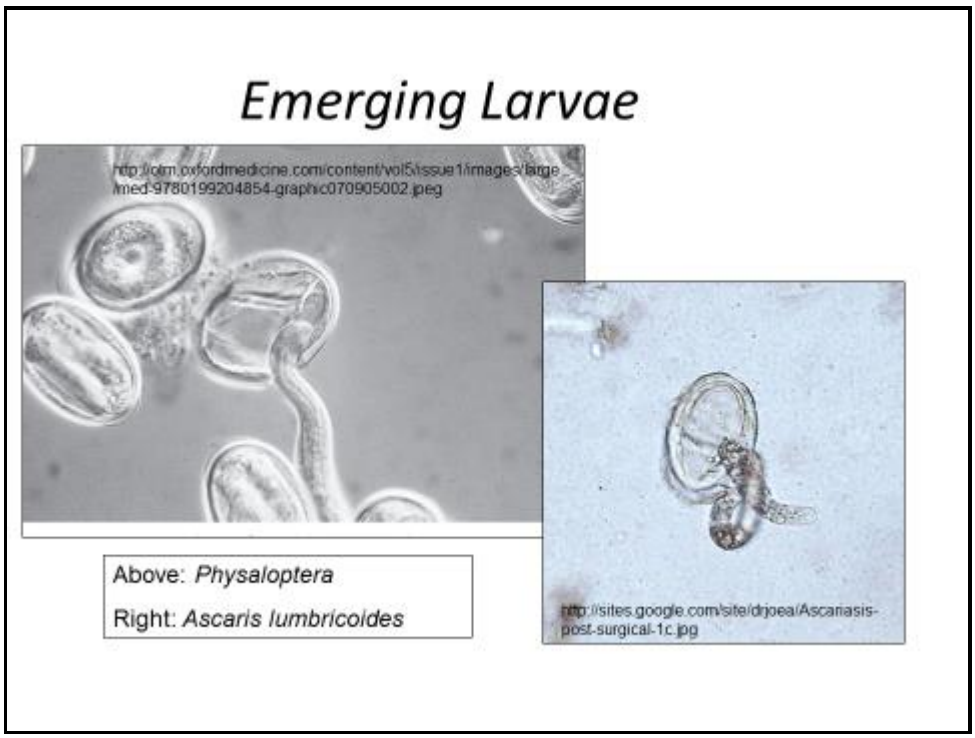

Figure 14: Comparison of emerging *Ascaris* and *Physaloptera* larvae

It is also equally possible that this result was due to contamination in the Veterinary lab, and so judgment was reserved until additional extractions and PCRs could be run on the retained and rehydrated material stored in the ancient lab.

PCR Results – Ancient DNA Material

In order to test the replicability of the results, new extractions on the originally rehydrated material, were performed, submitting them to the same protocols as outlined above and obtaining the same results, including the problematic *Contracaecum spiculigerum* sequence. (Cleeland, et al. 2013).

Additional Primer Design

Additional primers were designed in an attempt to increase the length of our fragment, as longer fragments provide stronger taxonomic assignments. The same PCR reaction recipe was used and the same 60 cycle cycling parameters with the exception of using 58ºC for the annealing temperature rather than the 52ºC used for the Asc 6-9 primers. With the data from several PCR reactions using three different primer sets (see Tables 3-6 above) and the three extractions allowed the construction of a consensus sequence of ~190bp for the 18S gene. There is a 28bp gap, representing about 15% of the consensus sequence. A BLAST search returned an 85% coverage (representing the gap area) and a 100% match to the following organisms: *Physaloptera* sp. SAN-2007; *Contracaecum spiculigerum*, both having the highest bit scores of 191 and E-values of 2e-45. Additional organisms identified as an 85% coverage and 100% match was *Physaloptera turgida* with a bit score

of 185 and an E-value of 8e-44, with bit scores of 180 and E-values of 4e-42 are *Turgida torresi* (also a Physalopterid and the only species in the genus *Turgida,* Ortlepp considers this genus and species to be synonymous with *Physaloptera torresi* (Ortlepp 1926) and *Physaloptera* sp. SAN-2010, having the same bit score and e-values but only an 83% coverage and 100% identity was *Physaloptera thalycomys*.

These results are fairly robust, with one exception, the continual inclusion of an Ascarid, *Contracaecum spiculigerum* in the identification results. If the Zape organism were an Ascarid as originally assumed, stronger results for similar organisms such as *Ascaris suum*, *Ascaris lumbricoides* or even the other *Contracaecum* species would be expected, but this is not the case. It does not make phylogenetic sense, which is one of the criteria of authenticity for ancient DNA work and requires investigation (Pääbo, et al. 2004).

Phylogenetic Tree Construction

Because of the lack of phylogenetic sense, published sequences for the 18S gene for *Ascaris*, *Contracaecum*, *Turgida*, and *Physaloptera* were pooled and aligned in Mega 5 (Tamura, et al. 2011). The *Contracaecum spiculigerum* sequence was a 100% match to *Physaloptera* sp. SAN-2007, but differed significantly from both the other *Contracaecum* and *Ascaris* sequences. The Zape 23 sequence was added and a Neighbor-Joining Tree Algorithm with 1000 bootstrap reiterations was performed (Figure 15). The results robustly separated the Physalopterids (*Physaloptera* and *Turgida*) from the Ascarids (*Ascaris* and *Contracaecum*) with a 95% confidence. It further differentiated the

Ascarids with 99% confidence between genera. For the Physalopterids, the tree separated the avian adapted species from the mammalian adapted species with a 90% confidence and with 86% confidence grouped the remaining sequences with the mammalian adapted species, including *Turgida torresi*, the Zape sample, and the anomalous *Contracaecum spiculigerum*. This suggests that *Contracaecum spiculigerum* is either more closely related to the Physalopterids than the Ascarids or that it is misidentified and is actually a *Physaloptera.*

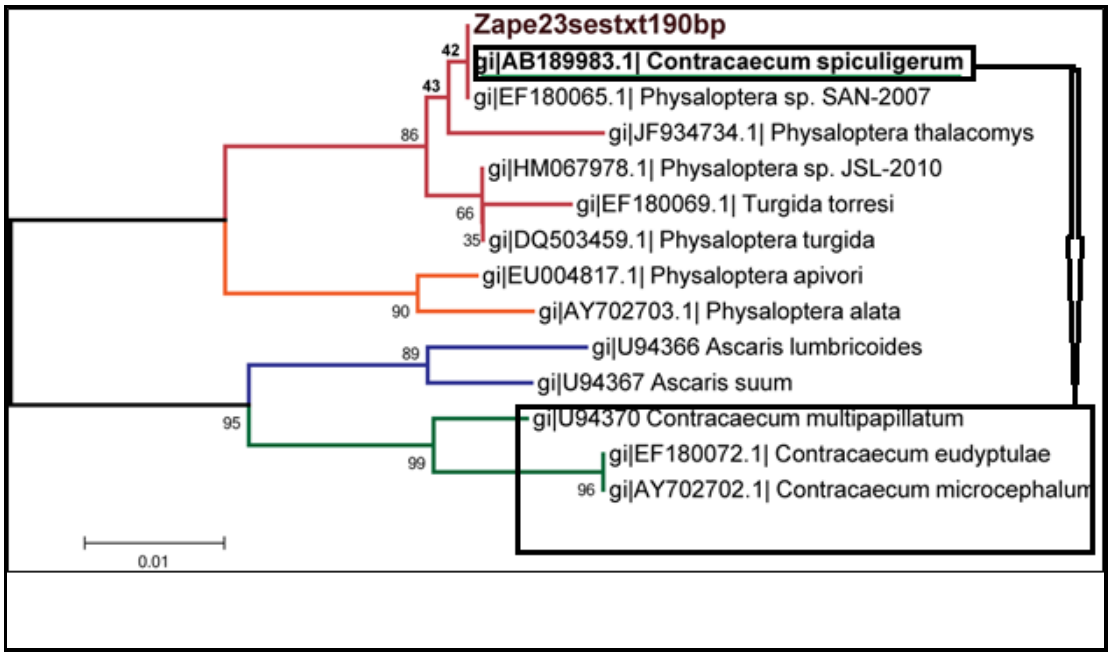

Figure 15: Zape 23 Neighbor-Joining Tree, 1000 boot strap reiterations, Green represents *Contracaecum spp*. Adapted from Cleeland et al. (2013).

In order to assess what these findings might suggest, a review of the documentation in the NCBI Blast database was conducted. It was learned that the *Contracaecum spiculigerum* sequence was a direct submission obtained from a juvenile specimen. The sample was recovered from a survey of raccoons in Japan in which *Physaloptera* were identified in all samples and *Contracaecum* in four. The report associated with the samples did not include a

discussion of the *Contracaecum* sequence and there were no sequencing was obtained from any of the recovered *Physaloptera* specimens not for the report or a direct submission (Sato and Suzuki 2006). Based on the difficulty of identification by morphological methods for many juvenile specimens, it is suggested that the sequence attributed to *Contracaecum spiculigerum* as a direct submission in the database is in actuality a juvenile *Physaloptera*. This conclusion also makes phylogenetic sense. By rejecting the *Contracaecum* as a misidentified *Physaloptera*, a robust assignment of the Zape sequence as a *Physaloptera* species adapted to mammalian hosts remains (Cleeland, et al. 2013).

An additional point of interest in the phylogenetic tree building analysis, is that there appears to be a distinct branching between mammalian associated *Physaloptera* and those associated with avian species (Figure 16). Further sequences from known host contexts are necessary to determine if this is a legitimate differentiation among *Physaloptera*.

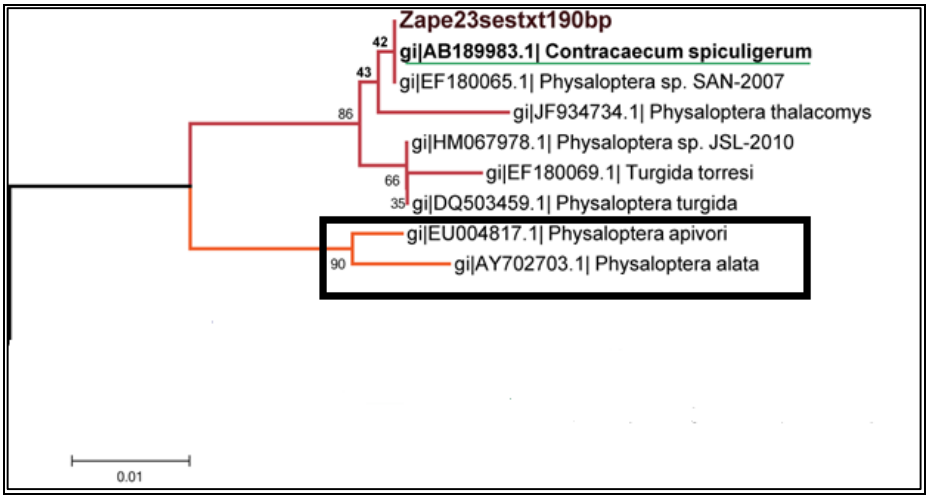

Figure 16: Phylogenetic tree showing the division between mammal associated and avian associated (boxed) *Physaloptera*. Adapted from Cleeland, et al. (2013).

Difficulty in Diagnosis of Physaloptera Infection

Physaloptera eggs are very similar to decorticated *Ascaris* eggs in appearance (Hira 1978). Several researchers note that this could be problematic in diagnosis. *Ascaris* is a very common parasite of humans both prehistorically and in modern populations A *Physaloptera* egg could very easily be misdiagnosed as a decorticated *Ascaris* egg. *Physaloptera* larvae are also often confused with *Ascaris* larvae (Fain and Vandepitte 1964; Hira 1978; Leiper 1911; Vandepitte, et al. 1963). Eggs of *Physaloptera* are also few in number and relatively heavy, so they may not be captured in a flotation protocol, although a Sugar Solution Flotation has been recommended by veterinary parasitologists (David and Lindquist 1982; Kazacos 2010). Females may not produce a large number of eggs; there is little information on the number of eggs produced, unlike *Ascaris*, which produce up to 200,000 eggs a day (Bogitsh, et al. 2013). It is suspected that adult *Physaloptera* are present in relatively small numbers, unlike *Ascaris*, which can be present in rather large communities. A recent study identified eggs attached to males, and may be purely accidental, or as the authors of the report suggest may be a method of egg dispersal, leaving the host body attached to a dead or expelled male (Oliveira-Menenzes, et al. 2011; Olsen 1986; Ortlepp 1922; Schell 1952)

Importance of Physaloptera Infection in Humans

The first case of human physalopteriasis was identified in 1902 by von Linstow in the Caucasus Mountains of Russia, and the species was described and named *Physaloptera caucasica* (Morgan 1945). Leiper identified additional infections in 1908, based on size differences, Leiper named this new human species *Physaloptera mordens* (Leiper 1908). Travassos reclassified *Physaloptera caucasica* to a new genus *Abbreviata* (Ortlepp 1926). In 1926, Schultz redescribed *Physaloptera caucasica* and gave a clearer description of the species (Morgan 1945). Ortlepp in the same year reexamined the two human species *P. caucasica* and *P. mordens* and determined that the morphological differences were too minor to justify two species and *P*. *mordens* became synonymous with *P. caucasica* (Ortlepp 1926). *Abbreviata caucasica* is also synonymous with *P. caucasica*, but both names should be searched in earlier literature. Fain and Vandepitte (1964) suggest that **if** the infection is truly as rare in humans as suspected from the literature, then it does not justify a specifically human species. Because *Physaloptera caucasica* is also found in non-human primates such as gorillas and baboons, they suggest that the infection in man is accidental and zoonotic in nature, with other primates acting as the natural reservoir for the parasite (Fain and Vandepitte 1964).Leiper (1908) states that the infection is relatively frequent in Central Africa and Fain and Vandepitte (1964) suggest this is due to the close ecological interactions between humans and primates. Whether or not *Physaloptera* infections are human specific or zoonotic in origin, they represent a serious health risk to

humans. Adults seem to weather the infection with less debilitating effects, but Nicolaides, et al. (1977) report on the infection of an eleven-month-old Australian infant with third stage infective larvae probably of a species specific to bandicoots, which caused bowel infarction and perforation, resulting in gangrene of the intestine. Had surgical interventions not been immediately available, this infant would not have survived.

Cases of human physalopteriasis have been reported from Africa, Central America, India, Russia, South America and North America (Anderson 1988; Apt, et al. 1965; Cleeland, et al. 2013; Fain and Vandepitte 1964; Fugassa, et al. 2007; Fugassa, et al. 2006; Leiper 1908, 1911; Lleras and Pan 1955; Morgan 1945; Nicolaides, et al. 1977; Ortlepp 1922; Ortlepp 1926; Schell 1952; Vandepitte, et al. 1963). Prehistoric diagnosis of *Physaloptera* infections have been potentially noted in two Argentine cases one a suspected canid and the other a human (Fugassa, et al. 2007; Fugassa, et al. 2006). The results reported here are the first confirmed case of prehistoric *Physaloptera* infection in a human subject employing molecular based tools (Cleeland, et al. 2013). These findings along with modern cases and the known difficulties in identifying *Physaloptera* infection suggest that this condition is more prevalent than previously believed. Because current treatments of the common helminths may prove ineffective for *Physaloptera* infections, the condition may remain active and occult for long periods of time. The symptoms of physalopteriasis are not unlike other gastric ailments. Vomiting, sometimes bloody, diarrhea, sometime bloody or black and tarry stools, abdominal and epigastric pain, chronic

ulceration and secondary infections are all possible (Apt, et al. 1965; Bogitsh, et al. 2013; Campbell and Graham 1999; Cleeland, et al. 2013; Fain and Vandepitte 1964; Leiper 1908, 1911; Lleras and Pan 1955; Vandepitte, et al. 1963).

Physaloptera spend time in intermediate arthropod hosts, such as grasshoppers, where they become infective third stage larvae, subsequently ingested by predators and/or definitive hosts. They also may make use of parenteric hosts such as snakes or frogs, where they can survive but not mature (Apt, et al. 1965; Basir 1948; Cawthorn and Anderson 1976; Cleeland, et al. 2013; Fain and Vandepitte 1964; Gray and Anderson 1981; Hobmaier 1941; Irwin-Smith 1921; Morgan 1945; Nicolaides, et al. 1977; Oliveira-Menenzes, et al. 2011; Olsen 1986; Ortlepp 1922; Petri 1950). Human entomophagy – the consumption of insects for food, has a long history and includes those insect hosts most probable in the lifecycle of *Physaloptera* (Callen 1965; Cort 1942; Gahukar 2011; Itterbeeck and Huis 2012; Rabenheimer, et al. 2014; Ramos-Elorduy 2009; Sutton 1995). In archaeological sites where evidence of entomophagy are found, *Physaloptera* infection should be considered. In modern cultures where the entomophagy are known, *Physaloptera* infections should be ruled out as part of the differential diagnosis of abdominal illness. Physaloptera infection can still be found among non-human primates in close association to people. This would suggest that the infection is sustainable and of potential risk to contemporary human groups (Bundy 1988; Campbell and Graham 1999; Flynn and Baker 2007; Hahn, et al.

2003; Johnson-Delaney 2009; Mbora and McPeek 2009; Murray, et al. 2000; Mutani, et al. 2003; Weyher, et al. 2006).

Physaloptera can be found in co-infection with other helminths and these other helminths, specifically the very common *Ascaris* can mask a *Physaloptera* infection. Ingestion of insect hosts can also be accidental through the grinding of grain along with insects. So generally wherever insect vectors are routinely encountered by humans, physalopteriasis should be considered. As noted above, the consequences for infants or children may be far more severe than for adults. Symptoms could also be attributed to other gastrointestinal illnesses which manifest similar symptoms such as dysentery.

Next Generation – Whole Genome Sequencing Results

Two different samples from the Zape coprolite were originally run as whole genome shotgun samples on a 454 pyroseqencer for examination of the gut microbiomes of prehistoric humans from the El Zape site *La Cueva de los Muertos Chiquitos*, a rock shelter site in Durango, Mexico along the El Zape river (Brooks, et al. 1962). The original runs resulted in 98% reads assigned to bacteria and less than 2% for each sample that include sequence reads for all other organisms contributing to the sample composition. The heavy bias toward bacteria is common for NGS WGS studies (Carpenter, et al. 2013). From morphological and targeted PCR analyses it is known that parasites are present in the Zape 23 samples, therefore, it is possible that some of the non-bacterial reads will be assigned to parasites. These reads were analyzed for two

purposes. First, to confirm the morphological and targeted PCR results and second, to identify additional parasites within the sample.

Reads are generally short sequences and as noted above, longer read lengths are more informative and more readily identified for taxonomic purposes (Wommack, et al. 2008). Related to read length, query coverage is also important in obtaining robust assignments. While it is possible to get 100% matches of very few nucleotides, with additional nucleotides, the assignment may change and the greater the query coverage the better the match (Newell, et al. 2013). With these considerations, a set of filters were used to whittle the dataset to those reads expected to provide the most robust information.

The first filter was to discard all reads with a bit score of less than 60. This is an arbitrary cutoff value. Researchers may choose whatever cutoff they desire. Ideally, bit scores provide an evaluation of the goodness of fit, so the higher the bit score the better the assignment. Bit scores should be comparable across databases. More information on bit scores can be found at: http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html.

The second filter was to discard all reads that were less than 100 bp in length. As noted above, the longer the read, the higher the probability of a good match. Related to length is the third filter, that of query coverage greater than 50%. This was determined by dividing the number of nucleotide matches by the length of the read. All reads with less than 50% query coverage were discarded.

The final parameter was an identity match of 95% or greater, all assignments below that cut-off were discarded. Nine reads remained at the end

of all the filtering from two Zape 23 sub-samples (see Table 11). These represented the most robust taxonomic assignments from the nematode specialized Nembase4 Expressed Sequence Tags (EST) database. Nembase4 hosts EST information for 62 nematodes, 11 of which are zoonotic to humans and 10 that are human specific (Elsworth, et al. 2011). Notable human specific parasites are missing, such as, *Enterobius vermicularis*, *Trichuris trichiura*, *Ancylostoma duodenale*, as well as the previously identified parasites for Zape 23 *Physaloptera* spp., and *Dipylidium caninum*.

In order to test the strength of the assignments provided by Nembase4, these nine reads were processed through a series of regular and specialized searches on the publicly available national genetic database NCBI, using the Blastn search function (Altschul, et al. 1990). Searching against the human genome, the canine genome and nematode genomes returned no matches and thus those three searches were discarded. The results of the other searches are found in Table 11.

Table 11: Final results of NEMBASE4 search, after filtering.

Read #	Read Identification	Sequence	Nembase4 assignment
1	FFTBGPP03C0KNR	accaatgaaataagaatcaaaatgatttcaaaagctccccagggggatccctgggtggcgcagcggtttggcgcctgcctttggcccagggcgtgatcccggagacccgggatcg	*Ancylostoma Caninum*
2	FFTBGPP03DIJP2	Agattaaccgctgcggtcagacgctgcaactgttgcgggagaatataatagggcggcatcaggtaaatcagtttgccaaaaggccggatccatacccccctgttcgacaaagaatttttgcagcgctgccatattcaccggacgagtggtttcgaccacgccgattgcccccagtacgcgcacatcggcaa	*Brugia malayi*
3	FFTBGPP03C17KE	Gagctgcacgacgccgaactgcctgtggaagtgtggctggtggcaagttccagcgaagaggtgggattacgcggcgggcaaactgccacccgcgcggtgtcgccggatgtcgccattgtgcttgatactgcctgctgggcgaaaaactttgattatggcgcagctaaccatcgccagattggtaacgggccgatgctggtgttaagcgacaagtcactgattgcgccgccaaaa	*Strongyloides stercoralis*
4	FFTBGPP03DB6L9	ggaacttagcataataatgcctttaatcattttctgcttaattttgctttcgcatttttgcagatcttctgccggattgtgcgacgtttgtaatatcacgtcgaaaccttcttcttccgctttggcggtgatggcatgcaaaacttcagagaaaaacggattacccgccgtagttttggt	*Caenorhabditis briggsae*
5	FFTBGPP03C1SYG	gacatgggcaaccggaagaggtcgctggtatggtcgcatggttagcagggccagaagccagttttgttaccggcgcgatgcataccattgatggcgcgtttggcgcataaccgactacgctcaattaagcccagccacctatccatgatgtctgggctttgt	*Ancylostoma caninum*
6	FGSU1FY06HAIXJ	Gtgatcgaagcgaaagaactgaccaaaaaatttggtgattttgccgccaccgatcacgtcaactttgccgttaaacgcggagagatttttggttgctggggccaaacagcgcagggaaatcgaccacctttaagatgatgtgcggtttgctggta	*Onchocerca volvulus*
7	FGSU1FY06G53IC	gcaaaccgcgtgcttccgccagttccaccgcagcgtgcagatagcttttcttcgtccggcctgccggtgcggatcagctcgagaaaatagcgatccgggaagtgttcttcataaaacgcgacacactcatctaccagcgcgctgttaccacgcaaaagactgcgtccaacgtcgcccatgcgccccgccggaaagaaagatcaacccttcatttaatt	*Strongyloides stercoralis*
8	FGSU1FY06G3BG2	gcaaaccgcgtgcttccgccagttccaccgcagcgtgcagatagcttttcttcgtccggcctgccggtgcggatcagctcgagaaaatagcgatccgggaagtgttcttcataaaacgcgacacactcatctaccagcgcgctgttaccacgcaaaagactgcgtccaacgtcgcccatgcgccccgccggaaagaaagatcaacccttcatttaatt	*Strongyloides stercoralis*
9	FGSU1FY06G1IK6	ccgttttcaccttacttccggttacgccaccagccgacaatcgctgcggtaataattcccgcaaggatcggtgctgccaggtcgtgccagaaagtcatggcaaactgcgcgagcgtcatatagccgccttgtgtgtaagttcacagagatattgcaattgcctccggataagtaagaggagattgcactatgcaaatgcagcatctgatggttggctatcctaagtactaccaaacggccgattatgcgttgaggctttcagtgatggctga	*Onchocerca volvulus*

Of the nine reads that met all filtering criteria, sequences two through nine all assigned best to *Escherichia coli*, while the other assignments are very

close, the match to the bacterium was the best match. To determine whether or not this represents an accurate assignment would require additional sequencing using targeted primers for species specific sequences. Read number one was assigned as *Ancylostoma caninum*, the dog hookworm by Nembase4. The subsequent searches were all associated with *Canis familiaris* to different degrees, but all were stronger matches than the hookworm assignment. The 100% coverage and 100% identity via the RefSeq search provides a secure identification as this sequence belonging to *Canis familiaris.*

Table 12: Results of BLAST searches for Zape 23 NGS-WGS data.

Sample N0.	Regular Blast N	Restricted Blast N	EST	Reference Sequences	Nembase4
1	***Canis familiaris 85%/99%***	***Canis familiaris 85%/99%***	***All Canine tissues 78%-96%***	***Canis familiaris 100%/100%***	*Ancylostoma caninum 74%/95%*
2	***Escherichia coli 100%/99%***	*Monosiqa brevicollis 98%/97%*	*Cryptosproidium parvum 98%/97%*	--	*Brugia malayi 98%/95%*
3	***Escherichia coli 100%/99%***	*Gryllus bimaculatus 42%/97%*	*Haliocynthia roretzi and Strongyloides stercoralis 98%/97%*	--	*Strongyloides stercoralis 99%/97%*
4	***Escherichia coli 100%/100%***	---	*C. briggsae 73%/99% and 63%/97%*	--	*C. briggsae 74%/98%*
5	***Escherichia coli 100%/100%***	--	*Ancylostoma caninum 90%/99%*	--	*Ancylostoma caninum 90%/99%*
6	***Escherichia coli 99%/97%***	--	*Multiple hits 99%/95%*	--	*Onchocerca volvulus 90%/95%*
7	***Escherichia coli 100%/99%***	--	*Haliocynthia roretzi and Strongyloides stercoralis 100%/97%*	--	*Strongyloides stercoralis 100%/97%*
8	***Escherichia coli 100%/99%***	--	*Halocynthia roretzi and Strongyloides stercoralis 100%/97%*	--	*Strongyloides stercoralis 100%/97%*
9	***Escherichia coli 100%/99%***	--	*Multiple hits 50%/97%*	--	*Onchocerca volvulus 51%/97%*

Therefore, we were unable to confirm the findings of *Dipylidium caninum* identified by Jiménez, et al. (2012), nor the *Physaloptera* finding from this study and reported in Cleeland, et al. (2013). While these results are disappointing, they are not surprising. Non-bacterial genetic sequences represent between 1 and 2 percent of the sequences read during NGS (Carpenter, et al. 2013). From morphological analysis a single species was identified – *Dipylidium caninum* (Jiménez, et al. 2012). Targeted PCR identified *Physaloptera spp*. (Cleeland, et al. 2013) Neither of these species are in the specialized nematode database housed at Nembase4 (Elsworth, et al. 2011). In the NCBI database, there are 12 sequences for *Dipylidium* and 15 for *Physaloptera*, all relatively small in length, 326bp – 2406bp, plus two 14,296 bp mitochondrial DNA genome sequences for *Dipylidium* and 320bp -- 1771bp for *Physaloptera* (Altschul, et al. 1990).

It should be noted, that an inability to identify parasites in the NGS reads, does not suggest that parasites are not present. Two factors in particular affect successful matching in this regard, first, the paucity of nematode genetic information in the databases, even those specializing in nematode genetics, and second, the nature of shotgun sequencing is such that random genetic fragments are captured and amplified. The sequences obtained from the reads may correspond to genetic sequences not included in the databases, even if other sequences are available for those species.

Of particular interest in the results is the 100% match to *Canis familiaris.* This could indicate one of two scenarios. First, the coprolite belongs to a dog. Second this is molecular confirmation of humans ingesting dogs as food without intact evidence of dog remains in the coprolite itself. Previously, the discovery of an intact canine cranial condyle encased in a human coprolite from Hinds Cave, Texas was the first molecular confirmation of dog consumption using an intact bone (Tito, et al. 2011). Prior to the Hinds Cave coprolite, evidence was inferred based on butchered dog bone and historical references to the use of dogs as food.

Evidence for dogs living in close association with the inhabitants of *La Cueva de los Muertos Chiquitos* is sparse. Brooks, et al. (1962) indicate the recovery of coyote bones in the faunal assemblage and suggest the inhabitants were hunting coyote for food. Skeletal differentiation between coyotes and canines is problematic due to similar morphology and only molecular identification can securely differentiate between the two (Byrd, et al. 2013). The NGS read identified as dog, differs from the NCBI coyote sequence in only two nucleotides, a third nucleotide is shared with two dog sequences but not the third.

The NGS read identified to dog by NCBI, but *Ancylostoma caninum* in Nembase4 is identical in sequence to dog. This suggests that this sequence in Nembase4 is an error, resulting from a dog and not a dog specific parasite, not unlike the *Contracaecum spiculigerum* species discovered during sequence taxonomic assignment of the targeted PCR amplicons. Unlike the

Contracaecum case discovered during the targeted PCR, there is no associated publication with this sequence. The work was done by Washington University of St. Louis Genomic Sequencing Center as part of their contributions to the Nembase4 EST database, but there is no other documentation regarding the origin of this genetic sequence nor how it was processed. Therefore, it is not possible to resolve the status of this sequence with any certainty.

NGS is of use to parasitological studies, but it is suggested that future research should revolve around the use of targeted 18S rRNA or mitochondrial COI genes coupled with powerful blocking primers which will enrich the non-bacterial components of the coprolite samples by binding bacterial sequences and preventing their replication. The development of a robust targeted NGS methodology has applications for both archaeoparasitology, as well as, modern parasitology. As nearly one third of the world population suffers from at least one of the four primary Soil Transmitted Helminths (Stephenson, et al. 2000b; WHO 2014), swift and accurate identification of large numbers of samples will benefit modern parasitology in three specific ways. First, targeted NGS will allow the collection and processing of numerous fecal samples for a more accurate survey of parasite distribution globally. Second, targeted NGS will allow a precise characterization of the parasite community infecting individual humans, especially those that leave no morphological targets. Third, targeted NGS will allow a usable test by which to assess either the need for treatment or the efficacy of treatment.

Errors in the database will be encountered. It is inevitable. Bhatia, et al. (1997) published a cautionary note in 1997 suggesting that erroneous sequences in the NCBI database were a substantial issue and that there could be as many as 3 errors per 1000 nucleotides. Klenk et al. (combined with (Bhatia, et al. 1997)) in response to this article suggested that the errors were more on the rate of 1 in 5,000 to 1 in 10,000 nucleotides. The responding authors also made note of the fact that they kept a curated in-house database of reference sequences with which to compare their sequence data. Bandelt, et al. (2004) highlighted serious problems in the mtDNA database housed by the FBI, and argues that critical evaluation is essential to the maintenance of a high quality database, failure to do so results in missed clerical errors and poor laboratory procedures leading to mixed samples. Gelbert (1998) argued for the same. He acknowledges that predetermining database needs is likely impossible, but periodic analysis of the accuracy and usability of the database is important, especially to address systematic errors and to address gaps in database coverage. High throughput data as generated by NGS technology only compounds the database issues.

Wasmuth and Blaxter (2004) coined the term “neglected genomes” to discuss deficiencies in the national databases, where sufficient sequences are not available for phylogenetic reference, and thus unavailable for taxonomic assignment. The situation was present and well acknowledged by 2002 and the problem remains. The only way to address this inadequacy is to perform concentrated work on morphologically identified nematode species to obtain

both gene oriented sequences information and genomic level information. As these data begin to be systematically processed and included evolutionary relationships will become interpretable and taxonomic classifications can be refined. It will also increase the chance of fruitful assignment from high throughput NGS reads.

The expansion of genetic databases is essential foundational work, but there is little money for growing a database specifically. Therefore, it is suggested that all researchers utilize some funds to process positive control samples from morphologically known nematodes and sequence them. These sequences should be submitted to the national NCBI database. Likewise, it would benefit researchers to maintain an in-house database of verified sequences. Poinar, et al. (2001) created their own reference database of plants from the Lower Pecos Region of Texas, prior to cloning coprolites for diet from three Hinds Cave coprolites. Klenk et al. in (Bhatia, et al. 1997) also note the use of an in-house database.

The morphological and molecular work performed in this study accomplished its tasks in providing robust verification of a rare human parasite in a prehistoric human. It demonstrated, through document review, the potential health effect of *Physaloptera* to humans, especially children. This research highlights the need to consider such parasites today, as well as, in the past. This study suggests that knowledge of subsistence practices, such as entomophagy, or the rearing of dogs in close association with humans for transport or food provides information sufficient for pursuing rare parasites. It

also highlighted the need to consider occult parasites, such as third stage infective larvae which can and do cause severe human infection, but will not be discovered by normal clinical testing (Nicolaides, et al. 1977).

While reprocessing the NGS data previously generated was unable to confirm either, *Physaloptera* or *Dipylidium* in the Zape 23 coprolite, it did highlight a number of issues. First, both the national NCBI database and the specialized databases are essentially inadequate to provide taxonomic assignment for most nematode species. Second, errors exist in both the national database and in the specialized databases. Errors that are not always resolvable using additional documentation. Third, shotgun NGS is not the most effective approach when seeking genetic information from the less than 2% non-bacterial reads generated by a shotgun run. A well-developed targeted approach has far greater potential, if, and only if, suitable reference material is also generated. Fourth, a complementary methodology is the most productive, using both morphological analysis and molecular analysis. The extraction and amplification of the material examined morphologically is possible and is beneficial, providing tests by which to compare additional PCR results and as a way of verifying morphologically ambiguous specimens. Fifth, this study has demonstrated that molecular analysis can identify genetic information for organisms which are no longer intact or physically present in a sample.

Chapter Five: Conclusion

Archaeoparasitology investigates parasitism in prehistoric humans. Based on morphological examination conducted by (Jiménez, et al. 2012), the inhabitants of *La Cueva de los Muertos Chiquitos* were parasitized at a much higher prevalence than other sites studied from the American Southwest or the Colorado Plateau. While they identified a number of species from Zape, only *Dipylidium caninum* was identified from Zape 23. This study added the presence of *Phsyaloptera spp*. Both of these can infect humans and cause serious health consequences especially in small children and infants. As this site is named for the large number of infant and small child burials found there, it is a parsimonious suggestion that parasitism may be the cause of under-five mortality at this site, or a significantly contributing factor.

This study aimed to demonstrate that molecular methods were possible for coprolite material and that they can be effective and uniquely informative. This study began with a set of assumptions and a set of questions expected to be supported during this study.

Question one posited that authentic ancient DNA was present in the coprolite and could be captured. This question is supported, by the recovery of *Physaloptera spp*. sequences, bacterial sequences and a *Canis familiaris* sequence.

Question two proposed that the reference databases would be sufficient to allow taxonomic identification at both the species and genus level. This question is partially supported. Reference material was present that allowed the

identification of *Canis familiaris* to both the genus and species level, but failed to provide species level information for *Physaloptera spp.* and *Dipylidium caninum* was not identified at all.

Question three suggests that both PCR and NGS technologies can provide parasite genetic information. This is likewise only partially supported. PCR did provide information on *Physaloptera,* but the NGS data did not provide parasite data.

Related to question three, question four suggests that Whole Genome Shotgun Sequencing using NGS technology would provide parasite data. This was not supported. The overwhelming presence of bacteria in the samples could have swamped the less frequent parasite genetic information, coupled with inadequacies in both the national database and specialized nematode oriented databases could have contributed to this failure.

Question five proposes that molecular information will enhance archaeoparasitological research. This question is supported. The recovery of *Physaloptera spp.* adds a new and rare parasite to the known parasites impacting prehistoric humans. It also provides new considerations for the health of the inhabitants of *La Cueva de los Muertos Chiquitos*. The large number of children under five buried beneath floor A, in association with the coprolite Zape 23, suggests a health crisis, as proposed by Brooks and Brooks (1978). The findings in this study recommend that parasitism must be considered as a potential cause. This is also a recommendation made by Jiménez, et al. (2012).

Related to question five is question six, which suggests molecular archaeoparasitology can replace traditional microscopic morphological analysis. This is not supported. While PCR has provided new and unique information, it is far more robust as a combined methodology, the strengths of one assisting the other and vice versa.

This study highlighted a number of areas where foundational work is necessary, such as adding new sequences from morphologically identified parasite samples. And, a systematic method of identifying and noting errors in the databases. NGS has great potential to propel parasitology, both prehistoric and modern, forward, by the development of effective bacterial blocking primers and well-designed targeted yet universal primers for 18s rRNA or COI genes (Soe, et al. 2015). Shotgun analysis is not recommended for parasite surveying. Any methodology developed for small fragment aDNA, is usable for modern assays as well.

This study also highlighted a number of benefits provided by molecular analysis. The ability to identify genetic material even in the absence of identifiable physical remains. The ability to amplify and analyze very small amounts of material, such as the residue remaining on the microscope slides after morphological examination. This is especially important when dealing with ambiguous eggs or larvae.

The overall conclusion of this dissertation is that archaeoparasitology should use a combined morphological and molecular approach. Every attempt should be pursued to develop and refine a highly informative and effective

targeted NGS method, which also enriches the lower frequency organisms through the use of blocking primers. Routine practice should include processing of morphologically identified parasites for their genetic information, which should then be submitted to the national database for public use. Both techniques and results should be shared with both archaeological specialists and modern parasitologists, in order to better define and understand human parasitism across time and space and its implications for human health and subsistence.

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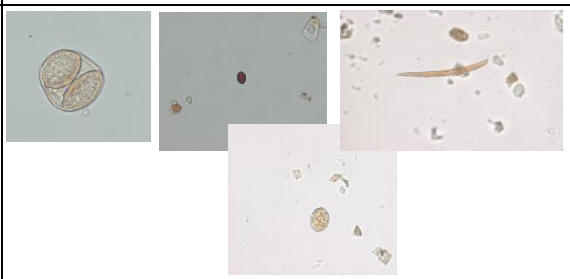
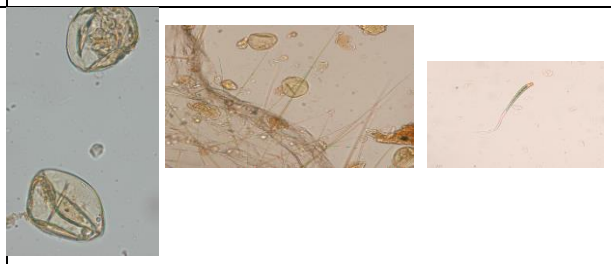
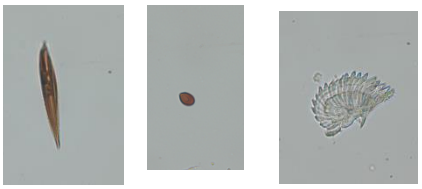
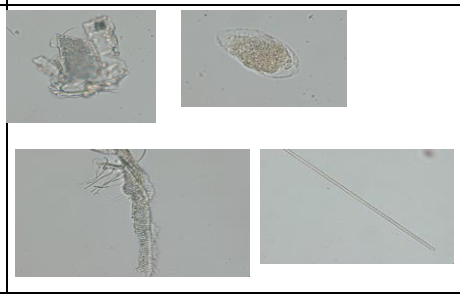
Appendix A: Timeline of aDNA and Parasites

Table 13: Appendix A: Photos Taken During Microscopy for Zape Samples

7	Sample ID	Flotation	Sedimentation	Photos taken
1	Zape 28	Pine Pollen; Fungal spores x 4; Seed pod, or elongated fungal spore; unknown structures x 6	balantidium coli (33um and 35um), poss taenia (35um)	
2	Zape 29	Unknown parasite egg x 49; Hair; seed pod or elongated fungal spores		
3	Zape 25	Seed pods or elongated fungal spores x13; small round fungal spores x 4	degraded whipworm, giardia, entoemeba dispar or histolytica, physaloptera	No pictures. Same structure as those taken with other Zape samples
4	Zape 2	seed pods or elongated fungal spores; unknown structure (with hooks?); plant hair	coccidia	
5	Zape 4	seed pods or elongated fungal spores x 6		No pictures; same structures as those taken with other Zape samples
6	Zape 23	unknown structure (probably plant); seed pods or unknown spores; unknown parasite, unknown plant structure; hair		

#	Sample ID	Flotation	Sedimentation	Photos taken
7	Zape 3	seed pods or elongated fungal spores (lots); unknown structure (no pictures)		

Appendix B: PCR Results for Zape 23

Table 14: Appendix B: Zape 23 PCR/Sequencing Results

Sequence ID	Sample	Target Organism	Primers	Length	Blast ID	Query Coverage	Maximum Identity	Comments
Zape23e_Asc6	Zape 23	Ascaris	Asc6	58	Physaloptera sp	77%	84%	
Zape23b_Asc8	Zape 23	Ascaris	Asc8	35	Turgida; Physaloptera; Physaloptera; Contracaecum	100%	100%	Also, P. thalcomys 91/100; P. Sp JSL-2010 100/97, P. apivori 100/94; P. alata 91/94 and Spirocerca lupi 67/100.
Zape23d_Asc8	Zape 23	Ascaris	Asc8	34	Turgida; Physaloptera; Physaloptera; Contracaecum	100%	100%	Also, P. thalcomys 91/100; P. Sp JSL-2010 100/97, P. apivori 100/94; P. alata 91/94 and Spirocerca lupi 67/100.
Zape23b_Asc9	Zape 23	Ascaris	Asc9	40	S. lupi, Physaloptera Sp JSL-2010, P. Sp. SAN-2007, Cyrnea seurati, C. leptoptera; P. Turgida, C. mansion	100%	100%	multiple strong hits, next batch is 100% coverage with 98% identity, even an ascaris with 95% coverage and 97% identity

Zape23d_Asc9	Zape 23	Ascaris	Asc9	40	S. lupi, Physaloptera Sp JSL-2010, P. Sp. SAN-2007, Cyrnea seurati, C. leptoptera; P. Turgida, C. mansion	100%	100%	multiple strong hits, next batch is 100% coverage with 98% identity, even an ascaris with 95% coverage and 97% identity
consensus sequence Zape 23 Asc 8, Asc and Asc 6	Zape 23	Ascaris	Asc6,8,9	108	P. sp SAN-2007;Contracaecum spiculigerum	87%	93%	P. turgida 87/92; P. thalacomys 85/91; P Sp JSL-2010 87/91;Turgida torresi 87/91; S. lupi 83/89; P. avipori 87/87
Zape 23 Asc 8/9 concensus	Zape 23		Asc8/9	57	P. Sp San-2007, P. turgida, C. spiculigerum	100%	100%	P. Sp JSL-2010 100/98;Turgida torresi 100/98; p. thalacomys 94/98; S. lupi 96/95; P. avipori 100/93; p. alata 94/93 [Ascaris sp 82/94]
ZA23	Zape 23	Ascaris	Asc8/9	57	Physaloptera sp SAN-2007	100%	100%	
ZA23	Zape 23	Ascaris	Asc8/9	57	Physaloptera sp SAN-2007	100%	100%	
LC10R_Asc9_Z23	Zape 23	Ascaris	Asc8/9	32	multiple hits including Spirocerca lupi, Phys Sp 2007 and 2010, Cyrnew seurati, Cyrnea leptoptera, P turgida, cyrnew mansion	100%	100%	

LC1R_Asc9_Z23	Zape 23	Ascaris	Asc8/9	40	multiple hits as preceeding, as well as Setaria digita, onchocerca cervicalis and contracaecum spiculigerum	100%	100%	
LC1F_Asc8_Z23	Zape 23	Ascaris	Asc8/9	34	hysaloptera sp SAN-2007, P. turgida, Contracaecum spiculigerum	100%	100%	
LC10F_Asc8_Z23	Zape 23	Ascaris	Asc8/9	33	hysaloptera sp SAN-2007, P. turgida, Contracaecum spiculigerum	100%	100%	
LC1F and LC1R	Zape 23	Ascaris	Asc8/9	57	Physaloptera sp SAN-2007, P. Turgida, Contracaecum spiculigerum	100%	100%	also P. sp 2010 100/98; Turgida torresi 100/98 Ascaris suum 82/94; Ascaris lumbricoides 87/92
LC10F and LC10R	Zape 23	Ascaris	Asc8/9	57	Physaloptera sp SAN-2007, P. Turgida, Contracaecum spiculigerum	100%	100%	also P. sp 2010 100/98; Turgida torresi 100/98 Ascaris suum 82/94; Ascaris lumbricoides 87/92
LC19F	Zape 23	Physaloptera	Phys243F	24	multiple 100%/100% hits			
LC17F	Zape 23	Physaloptera	Phys243F	24	multiple 100%/100% hits			
LC19F	Zape 23	Physaloptera	phys343F	35	multiple 100%/100% hits			

LC17F	Zape 23	Physalopter a	phys343F	32				
Zape 23 phys243/34 3 combined	Zape 23	Physalopter a	phys243/3 43	59	multiple hits 100%/100% including Physaloptera			
Zape23sestx t	Zape 23	Ascaris and Physalopter a	consensus	190	Physaloptera sp SAN-2007 85%/100%; contracaecum spiculigerum,P Turgida, P Thalacomys P sp JSL-2010, Turgida Torresi			28 base gap, 15% of 190 is 28.5 bp uses multiple primer sets Physa 243F/343R, Asc 8/9 and Asc 6/7
Zape 23combined	Zape 23		Asc6/7,asc 8/9	103	Physaloptera sp SAN-2007, Contracaecum spiculigerum and Physaloptera turgida	100%	100%	
Zape 23e	Zape 23	Ascaris	Asc6	53	Physaloptera Sp SAN-2007 and Contracaecum spiculigerum	100%	100%	
Zape 23	Zape 23	Ascaris	Asc 6/7	73	Physaloptera Sp SAN-2007 and Contracaecum spiculigerum	100%	100%	

Appendix C: First Contact: Archaeologists and Molecular Work

Abstract

Ancient DNA is a potentially powerful investigative tool for archaeological research. Similarly, archaeologists are powerful players in expanding and refining ancient biomolecular research. Archaeologists are the point of first contact with samples suitable for ancient DNA studies; they provide an important link in reducing modern DNA contamination, characterizing post-depositional processes leading to DNA degradation, and finding new ways ancient DNA may be used to address archaeological questions. This chapter explains the importance of archaeologists in ancient DNA research and provides idealized protocols for archaeologists. This chapter also addresses the ineffective dissemination of invitations to archaeologists to participate in ancient DNA studies and best practice recommendations from molecular researchers. This chapter provides information for both types of specialists in an attempt to improve both specialties involvement in ancient DNA research.

Introduction – Archaeological Prerogative

This appendix is written in three parts. Part one identifies the historical continuity of the archaeological prerogative in adopting and applying new analytic methods as they become available or in anticipation of their coming availability to answer archaeological questions. Part two addresses the information disconnect between molecular researchers who have called for increased archaeological involvement in ancient DNA research from the initial excavation but have routinely published in venues not highly utilized by archaeological researchers. And, part three provides a set of practical guidelines for archaeological field methods that are an idealized conception of best practices for material destined for ancient DNA analysis. It argues that incorporating techniques appropriate to the collection of samples for ancient DNA analysis is the prerogative of the archaeologist. This appendix argues that developing archaeological methods for ancient DNA analysis is simply the next step in archaeological methodology and presents no obstacle to or deviation from the historical progression of archaeological method development or a drastic rearrangement of current methodologies. This appendix joins previous calls from primarily molecular specialists for archaeologists to take an active role in ancient DNA research design and excavation. This appendix also expands the purview of the archaeologist to include the collection and documentation of contextual information, both environmental and cultural, which can only enhance ancient DNA analysis for future generations. A short overview of ancient DNA studies to date and the samples used is provided for general

background information. The focus of this appendix is not what can be done with ancient DNA, but rather how to improve the collection of samples suitable for ancient DNA analysis. Improving excavation, collection and storage techniques will only strengthen the authenticity and robusticity of ancient DNA analyses, which in turn will make ancient DNA analysis more cost effective and accessible to archaeological researchers. Therefore, this appendix will direct the reader to articles, which will provide more information on anthropological applications of ancient DNA, but it will not develop those lines of information in any detail. Instead, this appendix will present the case for the incorporation of techniques sensitive to ancient DNA sample collection into current methodology, based on historical method development and historical archaeological mandates. It will briefly detail the best way to excavate samples and it will then expand the data collection methodology to include the recovery of data necessary and helpful for the advancement of ancient DNA analysis as a powerful and informative investigative tool. In short, the archaeologist is the most important researcher in ancient DNA studies in three regards: first, as the instigator of collaborative associations that will address specific archaeologically rooted research questions and develop the research design; second, as the researcher with the expertise to properly excavate samples in the most appropriate contamination limiting manner; and finally, as the researcher who will be able to provide contextual data, both cultural and environmental, which will provide information that can only improve and advance future research.

Historical Archaeological Methodology Development and Practice Mandates

In 1951, Robert E. Bell, American Archaeologist at the University of Oklahoma wrote:

> "The advent of newly discovered data, not only in the nature of new materials but also in methods and tools for research, require a reinterpretation of much basic data. . .Because of improvements in observation and analysis of the raw data, the excavations and excavated materials offer much more information. . . What was considered unessential information ten years ago is now an integral part of most reports." Bell (1951:290).

American archaeology has lagged behind European archaeology routinely in both techniques and analyses. For example, Edward Deevey (1944) reports that in 1916 pollen analysis was routinely and widely used in Europe, but even in 1944 it was little used in American Archaeology. Even though analysis was not being undertaken, Eisley (1939) notes that American archaeologists found the results of ethnobotanical studies disappointing nevertheless many "dutifully saved" soil samples. Part of the problem was a paucity of trained archaeologists in North America forcing a reliance on outside specialists (Dyson 1953). The same problem existed for faunal analysis and even as late as 1970; John Mori warned, "North American archaeologists continue to devote minimal attention to the role of faunal remains (Mori 1970:387). Ford (1979) discusses the amount of data that had been tossed out during excavation; he notes the 1936 report on Newt Kash Hollow confirmed that much data was being discarded. In the early seventies, improved techniques including reducing the size of mesh used in dry screens, the incorporation of water screening and the beginnings of flotation all increased the recovery of data, including ever smaller remains (Lyman

1982:358). Lyman (1982:357) stated, “recovery can be controlled and modified by altering excavation techniques which in turn must be dictated by the questions being asked.” Swartz (1967:487-488) in speaking of archaeological objectives notes that the site report has taken on form and standardization as archaeology itself had developed and that it was the duty of every archaeologist in the field to “observe, record and collect data as completely and thoroughly as the appropriate techniques allow.” This material needs to be collected and documented even if it conflicts with the researcher’s more narrowed interests. Swartz (1967) divides archaeological work into two segments one in the field and one in the lab. Collection and documenting the excavation environment are the domain of acquisition and exclusively the domain of the archaeological specialist, while analysis is accomplished in the lab and can include a number of other specialists and disciplines.

In 1944, Alex D. Krieger urged archaeologists to provide “full description. . .in the hope that nothing of consequence will be overlooked (Krieger 1944:271).” We are currently entering a new phase of analytic potential, which requires slight modifications to excavation in the collection of samples suitable for ancient DNA analysis, and this being an acquisition-focused method is the distinct prerogative of archaeologists. It is time to draw together the varied calls to archaeologists and suggestions as to how best to collect samples for genetic analysis into a timely paper directed to the archaeological specialist and a generalized methodology, and presented in a media, which will reach archaeologists more routinely.

Calls to Archaeologists

In 1987, just two years after his cloning analysis of the ancient DNA of an Egyptian mummy, and a year after the breakthrough advance by Kary Mullis resulting in Polymerase Chain Reaction (PCR) capability (Mullis, et al. 1986b), Svante Pääbo published, “Molecular Genetic Methods in Archaeology. A Prospect.” In this article, Pääbo discusses the use of ancient DNA analyses as currently understood and how these might be beneficial for anthropological investigation. He ends the article with this quote:

> “A further prerequisite for any large scale endeavors in this direction is the systematic collection of samples of all tissue remains found at archaeological excavations as well as an intimate cooperation between anthropologists and archaeologists on the one hand and molecular biologists on the other. If this can be achieved, I believe that in the near future we will see fascinating new developments in this field (Pääbo 1987).”

In 1989, Pääbo published again, this time highlighting a number of important discoveries and considerations for working with ancient DNA. He outlines a preliminary version of the practices and protocols that become the Criteria of Authenticity for Ancient DNA analyses. He also highlights the number one issue in relation to obtaining authentic ancient DNA data – contamination – and suggests limiting those who handle specimens (Pääbo 1989). In 1991, Hagelberg and Clegg published the first paper using human bone for DNA analysis and they call for archaeologists and museum curators to “learn of the potential for genetic information in excavated skeletal remains and to develop appropriate methods for the removal and storage of samples for future study

(Hagelberg and Clegg 1991:49).” They also begin their paper by discussing the importance of minimizing contamination and in order to accomplish this, they provide the first list of considerations: use of gloves or forceps, and freshly excavated, unwashed samples. These considerations will remain in all subsequent recommendations.

Brown and Brown (1992) published in *Antiquity* an article that for the first time provides a set of guidelines for excavation of samples, with the aims of preventing contamination with modern human DNA and preventing the growth of fungi, bacteria and algae in samples. Their article also highlights the state of the discipline to that point and its applications to archaeology. Thomas (1993) stresses inter-disciplinary research between archaeologist and molecular biologists in bringing ancient DNA analysis to bear on archaeological problems. It approaches the subject, by answering the question from the archaeologist’s point of view, “What’s in this for us? Are these developments merely to advance techniques or will there be an attempt to apply new methods to questions of interest to the general archaeological community (Thomas 1993:1)?”

Gibbs (1993) published a review for archaeologists about the role of Ancient DNA in archaeology. In this paper she reiterates that the major problem in ancient DNA work is contamination with modern DNA and she follows with this warning to archaeologists: “The implications are clear: Archaeologists must exercise extreme care when excavating specimens destined for DNA analysis (Gibbs 1993:10).

Addressing these concerns as in-lab considerations Handt, et al. (1994) published a paper on the methodological concerns of ancient DNA work and offered criteria of authenticity for the laboratory management of the challenges of contamination and recovering genuine ancient DNA. Richards and Sykes (1995) report on authenticating ancient DNA as the methodological problems had remained. They note that in-lab protocols to help contain and manage pre-lab contamination have made it a somewhat manageable problem, but it could still be improved upon, by targeting pre-lab contamination events directly. In their paper and the study it reports, the authors tested both the potential to contaminate prior to the lab by ungloved handling of samples and the efficacy of decontamination methods in the laboratory. They found that gloved handling prevented pre-lab contamination to a large extent, and while in lab protocols such as bleaching and shot blasting the exterior of the sample, reduced or eliminated the contaminating DNA, this method is not applicable to all material types. Elimination of contaminate introduction in the field is vital.

Spigelman (1996) produced a short methodological paper for the Institute of Archaeology published by the University College London in which he provides a set of guidelines, potential samples and potential applications of ancient DNA work. Almost ten years pass before another paper is published relating to in-field contamination controls and this by Yang and Watt (2005). This paper was written specifically to archaeologists and was the first article of its kind published in a venue with the potential to reach a number of North American archaeological specialists, *The Journal of Archaeological Science.*

Their article is based on extensive research regarding contamination of ancient samples and decontamination methods. Their recommendations are sound and well explained and build upon earlier recommendations and observations. The newest call has been published by Morten Allentoft (2013). Despite the history presented above of calls from molecular researchers to archaeologists, there are still very few archaeologists participating as lead investigators and primary excavators of ancient DNA material. It begs the question as to why?

Deficiencies in Information Dissemination

As presented above, there were at least eleven calls to archaeologists to develop in field protocols with the express priority of limiting contamination of ancient samples with modern DNA, before this material ever reached the molecular lab. However, these calls were either ignored or never reached archaeological specialists, resulting in more and more curated materials were utilized in ancient DNA studies. Because the probability of contamination was high and more carefully excavated samples were not available, efforts turned to the development of in-lab decontamination protocols. Curated museum samples and other previously handled specimens are usable, but these are not optimal samples for two reasons. First, contamination with modern DNA is a tough problem, which can result in contamination issues far beyond the initial sample, controlling it in the field is the best method of controlling it period. Second, freshly excavated samples provide stronger more robust ancient DNA results (Pruvost, et al. 2007). Pruvost and colleagues published the results of their comparison of freshly excavated samples and stored samples and found

that the amplification results in freshly excavated samples were far better than those that had been curated. This makes sense. Once a sample is removed from its depositional environment, where it has reached some sort of homeostatic balance, it is exposed to an entirely new environment and as such, digenetic processes are restarted or accelerated, resulting in contamination with modern organisms and advancement of decomposition.

Given the sensibility of modifying excavation protocols to include guidelines for extracting some samples for possible ancient DNA analysis, why has it been met with such resistance? In discussions with archaeological colleagues, a few topics are often repeated regarding ancient DNA. First, it is far too expensive. Second, it is far too unreliable. Third, it is destructive. And, finally, because it is unreliable and destructive to precious unique samples, it is far too expensive to even consider sacrificing limited analysis funds on a project that might or might not offer any data and in the end would destroy irreplaceable samples. These are the very questions Thomas (1993) tried to address in his paper, concerned that ancient DNA developments would be useful for and accessible to all of archaeology, not just heavily funded and glamorized projects. It is a sad state of affairs when the same concerns issued in 1993 are still present in American archaeology 20 years later. One of the issues for American archaeologists is a deficiency in how ancient DNA information, regarding both applications and methodologies are disseminated in the US.

The flagship journal for North American archaeology is the SAA sponsored journal *American Antiquity*. A search of ancient DNA papers published in this journal originally returned 57 hits. A review of these articles found that two are mentions in 1992 under current research, one reporting on a study in Chile and one on a new lab and the recovery of DNA from deer bones. A third current research mentions a study on Chilean mummies. Ten book reviews have some mention of either ancient DNA or the use of modern DNA to extrapolate back to ancient populations. One overall review of the use of ancient DNA within anthropology was written by Connie Mulligan (2006), this follows a 2003 article by Berggren and Hodder (2003) which was not specifically related to ancient DNA, but argued that a disconnect between field archaeologists and later interpretation have created issues in the collection and later analysis of material. They argue that specialists should be present at and intimately involved in excavation and that knowledge of appropriate practices should be taught. Of the actual articles, the first ancient DNA study was published in 1998 and was conducted in a collaborative effort between archaeologists and researchers at the Mayo Foundation, examining skins and feathers (Borson, et al. 1998). A report mentioning DNA support for two lineages of squash appeared in 1999 (Fritz 1999). No other ancient DNA paper was published in American Antiquity until a 2006 article reporting on an analysis of ancient salmon remains (Cannon and Yang 2006b), in 2007 a report on northwest coast whale hunting followed (Losey and Yang 2007) and the final article was published in 2008 which mentions the discovery of Haplogroup M in

an ancient skeleton and the use of modern mtDNA to discuss human diversity in the New World, while its main emphasis was on the accuracy of radiocarbon dates at the earliest North American sites (Faught 2008). The only additional papers added after the initial search are two papers in 2011, a comment on Cannon and Yang's 2006 paper Monks and Orchard (2011) and the response from Cannon and Yang (2011). Cannon and Yang add an additional discovery concerning a problem with using curated materials, which has to do with limitations on suitable sampling based on earlier excavation methodologies, which may or may not have captured truly representative samples from various archaeological sites. They note a particular bias in the differential recovery of bones based on size (Cannon and Yang 2011).

A co-author of the 2006 and 2007 American Antiquity articles mentioned above, Dongya Yang, from Simon Fraser University in Canada coordinated the two part ancient DNA symposium at the 73rd Society of American Archaeology Conference in 2008, in Vancouver, British Columbia. These symposia were two of 321 posters and symposia offered over the five day conference comprising well over a thousand presentations and was attended by probably thousands of archaeologists and yet the symposia on Molecular Archaeology and the Archaeologist was attended primarily by the molecular specialists presenting.

Nearly all presenters at this conference argued that the most important step forward for molecular research would be in field collection utilizing contamination control procedures and yet overall few archaeologists were present to hear the calls. This is not the fault of the symposia coordinators, or

the presenters, after all, it seems a perfectly appropriate venue to reach the most archaeologists with archaeological specific recommendations. However, the sheer size of the conference and the number of presentations precludes an adequate attendance. Far more effective and efficient would be presentation at the smaller regional conferences where it is easier to attend multiple sessions and intermingling afterwards with the majority of attendees is a routine part of the event.

Additionally, many molecular papers are published in journals not routinely accessed by archaeologists. From informal discussions with archaeological colleagues, I was able to compile a list of journals routinely accessed or suggested as journals that should be routinely checked for new research. *American Antiquity* was the journal most often mentioned, followed by *Journal of Archaeological Science* and *Southeastern Archaeology*, *Plains Anthropologist* all receiving five or more mentions. Journals receiving 2 to 4 mentions included *Journal of Field Archaeology*, *Science*, *Journal of Archaeological Research*, *Journal of Archaeological Method and Theory*, *Antiquity* and *Journal of Anthropological Research*. Twenty additional journals received a single mention each.

Of the ten articles published between 1987 and 2005 that called for archaeologists to develop in-field contamination limiting excavation protocols only five were published in journals even mentioned by the archaeologists above, of these two were published in 1995 and 2005 in the *Journal of Archaeological Science* which came in third among the archaeologists who

offered suggestions for top journals . Three articles were in journals mentioned by single archaeologists and include *PNAS*, *Antiquity* and *World Archaeology*. *Antiquity* recently published an additional article by Allentoft (2013). The other five were in journals not mentioned and of relatively limited audience or accessibility (see Table 15). Therefore, it is possible to say that one reason American archaeologists continue to resist ancient DNA analysis is a lack of relevant, current information sharing in the primary archaeological journals. It should be noted, that no archaeologist or other researcher for that matter is limited to only a few journals, and often the journals used are topic relevant to current interests or projects. Therefore, I am not arguing a lack of access to information on ancient DNA research, but a lack of general interest access in the primary journals, which would expose researchers to current research projects and potential applications, regardless of the reader's particular interest in ancient DNA research for his or her own projects.

Table 15: Appendix C: Comparison of Journals Used by Archaeologists and Journals with Published Calls to Archaeologists, those in bold intersect.

Journals recommended by Archaeologists	Journals with published calls to archaeologists
American Antiquity 12/14 National	*Anthropologischer Anzeiger* 1987 by Paabo
Southeastern Archaeology 8/14 Regional	***PNAS* 1989 Paabo**
***Journal of Archaeological Science* 8/14 International**	*Proceedings of the Royal British Society: Biological Sciences* – 1991 Hagelberg and Clegg
Plains Anthropologist 6/14	***Antiquity* – 1992 Brown and Brown – 2013 Allentoft**
Journal of Field Archaeology 5/14	***World Archaeology* – 1993 Thomas**
Journal of Archaeological Research 3/14	*Canadian Student Journal of Anthropology* – 1993 Gibbs
Journal of Anthropological Research 3/14	*Experentia 50 Birkhauser Verlag* – 1994 Handt et al.
Journal of Anthropological Archaeology 3/14	***Journal of Archaeological Science* – 1995 Richards and Sykes – 2005 Yang and Watt**
Science 2/14	*Papers from the Institute of Archaeology* – 1996 Spigelman
Journal of Arch Method and Theory 2/14	
***Antiquity* 2/14**	
19 journals received one recommendation – Two of which correspond with a published call. . . ***PNAS* and *World Archaeology***	

Seamless inclusion

In an effort to demonstrate that the inclusion of the equipment necessary for the recovery of tissues for ancient DNA research and the techniques are a seamless inclusion into existing methodologies this section will include some practical considerations and some already well-established methods in addition to the specific suggestions for ancient DNA sample acquisition following the lead of Byers and Johnson (1939:190) who wrote, “In spite of the fact that these steps are well known, they are included here in an attempt to describe a routine of recording from beginning to end.” These two authors also offer support for the development of a specialized reporting and collecting protocol, noting that the fundamentals remain the same and when there are deviations, such as collecting a sample specifically for ancient DNA analysis, then “a careful description of the newly adopted process of recording makes it possible to work it into the general scheme (Byers and Johnson 1939:190).”

Ancient DNA is no longer in its infancy, curated samples have helped provide tests of the process and identifying and overcoming issues such as inhibition and contamination. However, it is now time for the process to expand into the archaeological field respectively. Freshly excavated samples provide the best amplification results (Pruvost, et al. 2007), proper field techniques also limit or eradicate exposure to contaminating modern DNA (Yang and Watt 2005), information gathered at the moment of excavation provides both cultural

and environmental contextual information which will enhance our understanding of preservation and behavior. This contextual information can provide clues for “in-lab” methods, which would be either beneficial or detrimental, based on accumulating knowledge of the depositional environments. And, finally, this is decidedly the domain of the archaeologist and as such, should be routinely taught and executed as a part of the archaeological mandate to preserve all information in the site as meticulously as possible through proper collection and recording (Champe, et al. 1961).

History of aDNA

Until recently much ancient DNA (aDNA) work has been exploratory and investigative. By this it is meant, researchers have tested its efficacy and suitability as well as its validity in studying the ancient past to delineate the boundaries of authentic ancient DNA recovery and its subsequent usability in answering archaeological and/or evolutionary questions. Because of this, many studies have been undertaken, utilizing previously excavated and currently curated samples such as the quagga (Higuchi, et al. 1984), Egyptian mummies (Pääbo 1985), and curated hair from Greenland (Rasmussen, et al. 2010) to name just a few. A nice review of successful and unsuccessful ancient DNA projects can be read in Paabo, et al. (2004) or Mulligan (2006) and more recent reviews by Rizzi, et al. (2012) and Kefi (2011).

Along with early successes, there were some outstanding claims of DNA recovered from amber preserved specimens (Cano and Borucki 1995; Cano, et al. 1993a; DeSalle, et al. 1992), but attempts to reproduce the results by Austin,

et al. (1997) drew the results into question. Improved methodologies and understanding of ancient DNA as well as its contexts have potentially returned amber to viable DNA sample options (Hebsgaard, et al. 2005; Martin-Gonzalez, et al. 2009; Viega-Crespo, et al. 2007; Viega-Crespo, et al. 2004) However, similar difficulties were discovered for claims of 20 million year old chloroplast DNA from Miocene fossil deposits (Golenberg, et al. 1990; Golenberg, et al. 1991; Kim, et al. 2004; Paabo and Wilson 1991; Poinar, et al. 1993; Watt 2005). The failure to replicate brought to light some issues relating to the properties of ancient DNA and led to the development of criteria of authenticity and a number of protocols to be used in the lab to account for these differences. For example, the use of amino acid racemization or collagen analyses as indirect measures of the preservation of DNA within a sample (Bada, et al. 1994; Collins, et al. 1999; Haynes, et al. 2002; Poinar, et al. 1996; Poinar and Stankiewicz 1999; Stankiewicz, et al. 1998). Recently, Collins, et al. (2009) and Kemp and Smith (2010) suggest amino acid racemization is only useful in specific contexts and not necessary in all ancient DNA studies. The use of methods to decontaminate samples using bleach, surface removal, and UV irradiation were also developed in efforts to minimize false positive reporting and authenticating that the DNA recovered from ancient material was legitimately that of the ancient material and not a more robust modern contaminate (Kemp and Smith 2005; Watt 2005).

Despite efforts in the laboratory to remove exogenous DNA contaminating ancient samples, it is at the stage of archaeological excavation

that the most advantage can be gained in protecting aDNA specimens from modern contamination (Allentoft 2013; Fortea, et al. 2008; Gilbert, et al. 2005b; Yang and Watt 2005). This dissertation argues that misconceptions about ancient DNA - both collection and analysis on the part of archaeologists and a lack of understanding of - both the contextual information and the best avenues to address large numbers of archaeologists on the part of molecular scientists have in essence created a void in the progression of the field. It is hoped that this dissertation spans that void and is able to offer strong reliable information appropriate for the archaeological specialist while also informing the molecular specialist in how to best connect with and collaborate with archaeology.

Archaeological Domain: Acquisition

Acquisition is the domain of the archaeologist (Swartz 1967). In this capacity, the archaeologist is the first to connect with potential samples. Acquiring samples requires only minor modifications to existing excavation procedures, a little more paperwork, and a consideration of storage and chain of custody for the samples. The archaeologist is of prime importance in ancient DNA research for the following reasons:

1. They have the specialized skill to identify, assess, excavate, and store samples in the field.
2. They provide the cultural context to the samples, which allows interpretation of the results within an anthropological framework, to increase our understanding of ecology, biology, and culture.

3. They provide the in situ environmental context of the depositional environment, the post-depositional processes, and the excavation environment.
4. They provide the research questions, which direct the genetic analysis at the lab level.
5. Their collection of preservation and environmental information on the micro and macro levels provides the molecular researcher with invaluable information regarding potential inhibitors, potential contaminants and other perhaps as yet unknown conditions, which can make preservation differ even from one end of a bone to another.
6. As experts in excavation methodology, it is the archaeologist who will ultimately refine the methodological process suggested by molecular researchers, to further improve the acquisition of preferential samples for genetic analysis.

The information, which follows, is primarily oriented toward the archaeological specialist. Information that is already common practice is presented here, in order to stress how seamlessly the addition of specialized collection and documentation desirable for ancient biological samples can be integrated into the existing practice, not to imply a lack of the current practice. Additionally, it is hoped that this paper demonstrates that the call for archaeologists to engage in ancient DNA collaborations and sample acquisition was first proposed very early in the development of ancient DNA technique, but due to limited audience exposure, these attempts have failed to have the impact

desired. A final consideration we hope to accomplish is to dispel some misconceptions regarding ancient DNA research in relation to its scope and practice.

Collecting Information

The inclusion of archaeologists in all phases of aDNA research is important. Archaeologists are the first to encounter the depositional environment and record their observations (Burger, et al. 1999; Child 1995; Gilbert, et al. 2005b; Haynes, et al. 2002; Hofreiter, et al. 2001a; Kaestle and Horsburgh 2002; Paabo, et al. 2004; Poinar, et al. 1996; Poinar and Stankiewicz 1999). Within these observations is information vital for inferring aDNA preservation, modern DNA contamination, and chemical inhibitors of molecular techniques.

The depositional environment may be the single most important factor in recovering aDNA (Burger, et al. 1999; Gilbert, et al. 2005b; Marota, et al. 2002; Yang and Watt 2005; Zink and Nerlich 2005). Cold and dry environments with a neutral to alkaline pH are ideal, but aDNA can be retrieved from other environments. Context is everything in archaeology, therefore, a detailed archaeological record of the depositional environment is invaluable for resolving post-depositional processes influencing aDNA preservation, both for specific studies and in general. In addition to resolving post depositional processes, good thorough contextual information will provide the molecular researcher information with which to determine how best to proceed in their extraction protocols. For example, Hofreiter, et al. (2001a) determined that the addition of

PTB to coprolite samples released the sugar cross-links and allowed the retrieval of the trapped DNA. Understanding that a sample was retrieved perhaps within a latrine, allows the researcher to consider the use of PTB in their extraction protocols.

We provide an example of an ancient DNA excavation record in the appendices. With regards to DNA preservation, factors would include pH level, ambient air and soil temperature, humidity, seasonal fluctuations, soil type and composition, levels of humic and fluvic acids, extent of bioturbation and infiltration by root systems (Gilbert, et al. 2005a; Marota, et al. 2002; Prangnell and McGowan 2009; Zink and Nerlich 2005). It is important to note that soil type and composition are critical because DNA once released from the cell will bind to clay when certain chemical conditions are present (Alvarez, et al. 1998).

A record of the sample's physical characteristics is required for exploring morphological associations with aDNA preservation. The development of these morphological indices will assist in selecting future samples for aDNA study (Gilbert, et al. 2005b; Haynes, et al. 2002). For one example, bone samples that are chalky and brittle are unlikely to retain DNA. Bone with extensive boring from diagenetic changes likewise may be compromised for DNA extraction (Gilbert, et al. 2005b; Haynes, et al. 2002). Conversely, perfect morphological preservation is not necessarily an indicator of DNA preservation as has been discovered with amber preserved specimens, which retain perfect morphological preservation but have proved virtually fruitless for DNA extraction and reproducible results (Austin, et al. 1997a; Stankiewicz, et al. 1998). Hair

provides a good source of mitochondrial DNA due to its hydrophobic properties A record of the sample's physical characteristics is required for exploring morphological associations with aDNA preservation. The development of these morphological indices will assist in selecting future samples for aDNA study (Gilbert, et al. 2005b; Haynes, et al. 2002). For one example, bone samples that are chalky and brittle are unlikely to retain DNA. Bone with extensive boring from diagenetic changes likewise may be compromised for DNA extraction (Gilbert, et al. 2005b; Haynes, et al. 2002). Conversely, perfect morphological preservation is not necessarily an indicator of DNA preservation as has been discovered with amber preserved specimens, which retain perfect morphological preservation but have proved virtually fruitless for DNA extraction and reproducible results (Austin, et al. 1997a; Stankiewicz, et al. 1998). Hair provides a good source of mitochondrial DNA due to its hydrophobic properties (Gilbert, et al. 2008b; Gilbert, et al. 2004) and recently has been a source for nuclear DNA as well (Amory, et al. 2007; Rasmussen, et al. 2010).

Archaeological implementation

There are a few traditional sample types in aDNA research; these include tissues such as bone, hair, skin, teeth, muscle or organs, plant remains, soils, paleofeces and artifacts covered with residues(Allentoft 2013; Gibbs 1993; Hofreiter, et al. 2001; Loy 1993; Paabo, et al. 2004; Wayne, et al. 1999; Willerslev and Cooper 2005a). The search for new and novel sample options is ongoing and it is important to remember that any organic matter has the potential to harbor fragmented DNA. The archaeologist is in a prime position to

identify new sampling options. A mock plan view with positions and types of samples to consider is provided in FIG. 17.

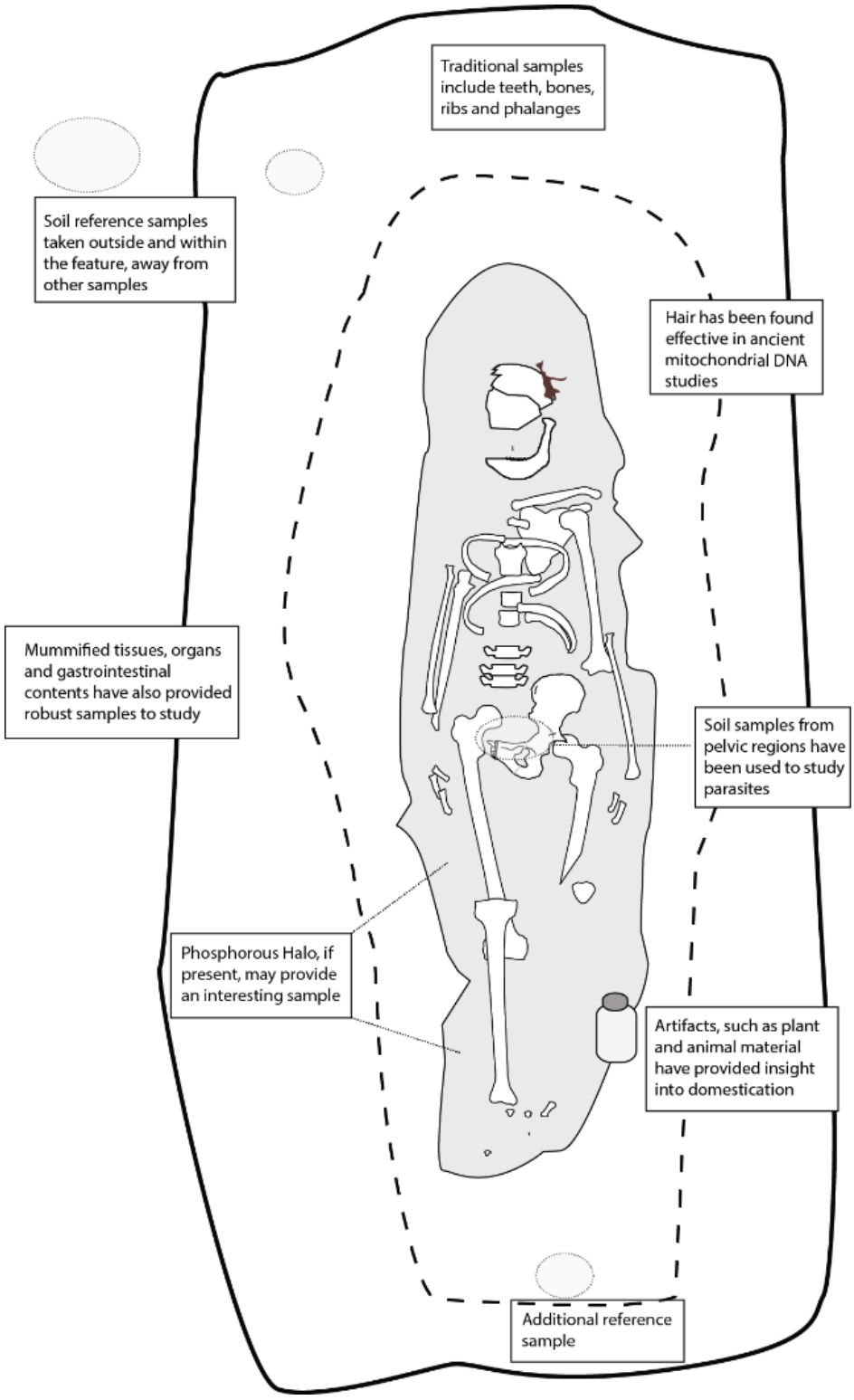


Figure 17: Appendix C: Idealized Excavation Plan View Highlighting examples of DNA Samples.

Archaeologists are the key to establishing and implementing contamination controls from the moment a sample is first encountered (Fortea, et al. 2008; Gilbert, et al. 2005a; Gilbert, et al. 2005b; Yang and Watt 2005). Freshly excavated samples provide better DNA recovery as discovered by Adler, et al. (2011) in a comparative study between freshly excavated and curated teeth and bone and earlier by Pruvost, et al. (2007). Therefore, it is ideal to protect the sample from unnecessary contact. Contamination can occur at any point along the continuum from excavation to processing in the lab. Using aseptic technique borrowed from medical practice, we are able to protect these fragile samples from the outside world (Dougherty and Lister 2004). We are constantly shedding DNA in skin cells, lost hair, and saliva droplets. This shed DNA may come from us or from any member of our biome, for example the bacteria that live on our skin or respiratory tract. Therefore, it is most important that the sample not be touched with bare hands. Latex or nitrile exam gloves should be worn when handling samples and the excavator should change gloves in between samples. Additionally, suspected organic samples destined for genetic analysis should not be tested against the tongue, a common field school lesson; the same capillary action that allows the organic material to stick to the tongue will draw in contaminating modern DNA. Surgical masks should be worn at all times to prevent breathing, sneezing, or coughing on the sample. The number of individuals excavating and handling the specimens should be restricted, ideally, to one person (Yang and Watt 2005). Limiting personnel lowers exposure to contaminating DNA.

Non-human contaminates should be considered, depending on the purpose of the study. For example, paleofecal based studies of diet can be contaminated from contemporary plants and animals. Cross contamination between paleofecal samples may be a concern (Yang and Watt 2005). A record of sample contact and treatment is ideal. There are several questions to consider. Who handled the sample? How the sample was retrieved, preserved, and stored? What equipment was used? An example of an aDNA excavation record is included in the appendices.

It is routine to wash archaeological artifacts and samples together often in the field lab and brush them free of depositional debris. Typically, such sample preparation should be avoided for aDNA analysis (Yang and Watt 2005). Samples for aDNA analysis should be individually bagged and isolated from other samples including other aDNA samples. Packaging should be as sterile as possible, utilizing sterile containers such as polypropylene conical lab tubes or sample bags. The sample should be carefully stored in a dry and cool environment and not opened until it reaches the genetic laboratory. Storage in a cool environment will retard fungal or bacterial growth. It is also important to limit condensation within the bag; this is a difficult process without cold storage. Desiccants and oxygen absorbers may provide additional aids for in-field storage, but their efficacy has not yet been tested. Ideally, these products would damaging condensation while posing no external contamination threat.

Reference samples are an important consideration. It is ideal to have DNA controls from all handlers of the sample, but not necessarily possible

(Gilbert, et al. 2008b; Oh, et al. 2010). While these modern reference samples will have a limited scope of use, they will likely require informed consent from the donor. Researchers should seek an appropriate Institute Review Board to evaluate their procedures on any genetic study that includes living human subjects, even if the collected control data will not be made available publically.

Reference soil samples can provide important intrasite genetic comparisons. These controls may be critical for a wide range of studies. In fact, soil samples are the primary implementation for emerging studies of molecular stratigraphy through metagenomics (Hebsgaard, et al. 2009; Willerslev, et al. 2003). With the budding, relatively affordable genome sequencing technologies, soil DNA fingerprints may become as common as ceramic, lithic, and faunal bone characterization. The current momentum of environmental genomics is literally and figuratively ground breaking, with substantial public and private funding (Simon and Daniel 2009; Singh, et al. 2009; Steven, et al. 2008; Tringe and Rubin 2005; Vogel, et al. 2009). Ancient DNA samples collected for stratigraphic comparisons are particularly sensitive to burrowing animals and contamination from sources as small as bacteria and pollen. Ideally, a fresh sample would be bagged immediately after exposure (Allentoft 2013; Fortea, et al. 2008; Pruvost, et al. 2007). An alternative method of collection is the use of a prepared pipe driven into the soil as a coring tube with each end immediately capped and sealed (Willerslev, et al. 2003). This may also be treated with a known bacterial spike in order to evaluate penetration of potentially contaminating DNA (Haile, et al. 2007; Hebsgaard, et al. 2009). Moist soil may

also result in continued bacterial and fungal growth after excavation. Depending on the research design, immediately placing the samples in a cell lysis buffer may be appropriate. These buffers help prevent bacterial and fungal growth while retaining DNA integrity. The OU Molecular Anthropology lab uses the following lysis buffer: 400mM NaCl, 10mM Tris HCL pH 7.5 and 100mM Na_2EDTA pH 8.2. This buffer has more EDTA than many conventional buffers to improve the stability of the samples when stored at room temperature for long periods of time.

It is important to reiterate that we present an idealized scenario. Most aDNA studies will deviate from this ideal in some fashion or another. For some research designs, these precautions are less of a concern. Samples of dense bone, for example, can be decontaminated by applying a bleach solution to the bone surface in the genetics lab (Kemp and Smith 2010). However, softer tissues have fewer options and the impact of archaeological practice becomes a great concern. As an additional example, the analysis of animal DNA or plant DNA, do not pose as great a concern for injury by modern DNA as do ancient human studies, which are severely compromised by modern human contaminates.

Curation

Curation should seek to avoid any further molecular deterioration, modern DNA contamination, and growth of bacteria and fungi. From the moment a sample is exposed, taphonomic processes likely accelerate. Ideal conditions would be dry, cool, and non-acidic, with each sample isolated from

contaminates. There are two goals of aDNA curation: 1) preserve the samples to suspend taphonomic processes, 2) prevent exposure to contaminating DNA.

Documentation and the Ancient DNA Field Kit

Until the advent of an effective flotation procedure some forty years ago, archaeologists rarely recovered micro fossil bone, or plant remains for analysis (Deevey 1944; Ford 1979; Lyman 1982; Swartz 1967). Today, faunal, pollen and environmental analyses are extremely sophisticated. Archaeologists routinely screen soils samples with both wet and dry methods and float samples in the lab to recover micro-artifacts and organic remains including seeds and bones. Excavation to recover ancient genetic material is merely the next step in this process. It does not require a major shift in practice; it simply requires a few specialized tools and a little extra paperwork. Because of excavation's destructive nature, it is standard archaeological practice to thoroughly document the site through detailed mapping and piece plotting of recovered material. It is also common practice to use specialized excavation reports for features such as storage pits in addition to the general excavation record. It is no different for aDNA specimens. Ancient DNA excavation should also include a specialized form with a map of the specimen recovered and other associated artifacts. Information that is useful includes an environmental history, soil and weather conditions at the time of recovery, and the process used in excavating. It is beneficial to note the names of all handlers of the specimens, the collaborators and labs that will be used, and the purpose of the samples. Documenting the manner of storage and the anticipated curation strategy on

this original sheet serves as a long-term reference to sample management. We provide an example excavation report in the appendices.

Unexpectedly, archaeologists may uncover artifacts ideal for aDNA studies; however, they may be unprepared to extract the artifacts in a way that is favorable for molecular analysis. A small and inexpensive aDNA field kit (FIG. 18) will prepare an archaeologist for sample extraction. Archaeologists may already use some of the items.

Ancient DNA Field Kit

- Exam gloves, latex or nitrile (if the excavator has a latex allergy)
- Zip seal sample bags
- Sterile scalpels
- Disposable palette knives
- Household bleach
- Reference sample material:
    - Options include buccal (cheek) swabs, saliva samples,
    - and Whatman FTA blood cards.
    - Informed consent forms, if necessary
- Reference Sample Material: **Depositional Environment:**
    - Sterile polypropylene conical tubes 15ml and 50ml, sample bags
- Documents: Ancient DNA excavation record (see supplementary data)

Figure 18: Ancient DNA Kit Components

Sample kits should be packaged separately (FIG. 2). Tools should be sterile. Surface DNA is sufficiently destroyed by a 15 minute soak in 3.0% (w/v) sodium hypochlorite, which is roughly equal parts of commercial bleach and water (Kemp and Smith 2005; Watt 2005). Metal trowels may be used after bleach treatment (Watt 2005; Yang and Watt 2005), but it should be noted that

bleach will corrode metal over time. Inexpensive plastic palette knives, which are sturdy and can be bleached and reused, are suitable alternatives. To limit contamination it is imperative that tools be cleaned or replaced between the collection of different samples. The aDNA kit may also contain materials for collection of DNA samples from the handlers of the ancient sample, which would be used if the project has informed consent. An example of reference sample preparation may be buccal (cheek) swabs and tubes of saliva or FTA Whatman blood cards (Yang and Watt 2005). Archaeologists should be able to obtain a sample kit or its components from their molecular collaborators.

Collaboration

One of the first questions to answer is whether or not the samples are to be outsourced to a commercial laboratory or whether an academic collaborative relationship is to be established with a researcher and his or her laboratory. Ancient DNA is different from forensic DNA. The geneticist should have a laboratory designed for aDNA studies, which follow a series of specific protocols (Hofreiter, et al. 2001; Kemp and Smith 2010; Paabo, et al. 2004; Wayne, et al. 1999; Willerslev and Cooper 2005; Yang and Watt 2005).

When the archaeologist leads quality control

If the archaeologist is also the principal investigator (PI) for the aDNA project, approaches to quality control should be considered. Ideally, duplicate samples that are de-identified to all but the PI are sent to the molecular lab for processing. This allows the PI to determine the consistency of the results. Independent laboratory testing is another option. In this approach, two

independent laboratories are sent the same samples and generate the data independently of one another. The results are then compared for consistency. Frequently, the laboratory serves as collaborators who will take on the responsibility of authenticating the data. For a thorough treatment of criteria of authenticity in aDNA work, see Paabo, et al. (2004). A collaborator should exhibit a verifiable record of compliance with these types of standards.

Research Questions

With all archaeological projects, research questions can guide the preparation and approach taken to excavation. Familiarity with aDNA research and its applicability to archaeological questions is important. For example, seeking to undertake a population study is not appropriate for an excavation that will yield a single specimen, but may be very applicable to a bison kill site or a communal cemetery. Single or small numbers of specimens may be good sources of information on diet and disease. The type of sample excavated may also provide an avenue of research, for example, paleofecal samples provide the opportunity to identify information on diet, environment, parasitism, health, and even the species of the host.

The feasibility of a study may be approached through pilot study. The preservation of aDNA is tenuous. Pilot studies provide a relatively cost efficient approach. A little experience with calculating probability is helpful in determining the feasibility of a study. For example, let us assume 40% recovery rate for aDNA is deemed acceptable given a project's budget, time and number of available samples. If the recovery rate is exactly 40%, a pilot project of only five

samples would have a 92.2% chance of at least one success. To explain, let x be the probability of being unsuccessful (in the example: 0.6) and t be the number of trials (in this example: five), then probability of at least one success in four trials is given by the equation: $1-\left(x^{t}\right)=1-\left(.6^{5}\right)=0.922$. If this pilot test fails to have a success, it likely means that the preservation rate will be less than 40% and a decision will need to be made whether to continue with the project or abandon it.

Conclusion

Archaeologists are a vital part of the aDNA collaborative network. As a discipline, archaeology possesses intricate knowledge of varied depositional environments, artifact taphonomy and specialized excavation protocols. Archaeologists are also primarily concerned with questions such as diet, disease, and group affiliation all answerable via molecular analysis. Contamination continues to be the primary confounding factor in aDNA projects, because degraded DNA is highly susceptible to being overwhelmed by more robust modern DNA sequences during laboratory chemistry, such as the Polymerase Chain Reaction (PCR). In order to address this issue, many molecular researchers have argued for excavation protocols that will permit the excavation of samples in a contamination-minimizing manner (Allentoft 2013; Brown and Brown 1992; Cipollaro, et al. 2005; Fortea, et al. 2008; Gilbert, et al. 2005b; Handt, et al. 1994; Hofreiter, et al. 2001; Paabo, et al. 2004; Pruvost, et al. 2007; Spigelman 1996; Thomas 1993; Yang and Watt 2005).

Archaeologists, as the point of first contact between archaeological specimens and modern technology represent the first line of defense in protecting degraded ancient samples from confounding contamination. In addition, archaeologists bring valuable information to the multidisciplinary aDNA project regarding deposition and preservation. Their environmental expertise and understanding of ancient cultures via the material record provides the potential to discover new sample material, new applications for aDNA analysis, and improved methods for interpretation.

Ancient DNA studies have progressed to a point where applying molecular methods can be a routine part of archaeological research. The archaeologist, as point of first contact, must be an informed partner in developing and actualizing aDNA research projects. Education and training geared toward planned excavations will allow archaeologists to manage unexpected aDNA sample opportunities efficiently and appropriately.

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