Part II - Parasite Remains Preserved in Various Materials and Techniques in Microscopy and Molecular Diagnosis

19. Molecular Paleoparasitology

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Molecular Paleoparasitology

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ANCIENT DNA: CONCEPTS

Molecular biology techniques have been used for more than two decades to recover and analyze DNA in biological materials preserved from the recent or distant past, thereby establishing ancient DNA as a line of research.

The term “ancient DNA” (aDNA) was first used to designate DNA fragments found in some type of preserved or fossilized biological material (Brown & Brown, 1994), such as human or animal bones, teeth, tissues, hair, and coprolites, insects preserved in amber, and plant seeds and other plant materials (including those found in plant collections and permafrost).

A more comprehensive concept for this term refers to any DNA vestige or trace from a dead organism or parts of it, as well as extracorporeal DNA (Herrmann & Hummel, 1994). Other terms involving the study of aDNA are also used, such as “paleogenetics” or “molecular paleontology” (Marota & Rollo, 2002; Loreille & Bouchet, 2003), or molecular paleoparasitology (Ferreira et al., 2000) and paleomicrobiology (Drancourt & Raoult, 2005), the latter specifically for the study of aDNA from parasite microorganisms that cause human diseases. Finally, “paleogenomics” (Poinar et al., 2006) involves the determination and analysis of long segments of nuclear aDNA, on the order of millions of base pairs (bp), or even complete mitochondrial genomes (mitogenomics).

ANCIENT DNA: HISTORY

The first attempts to recover biological material from ancestral remains specifically involved proteins. Protein residues were identified in different fossil remains, such as shells (Weiner, Lowenstam & Hood et al., 1976), stone tools (Loy, 1983), and bones (Ascenzi et al., 1985). However, the pioneering evidence in detecting ancestral proteins emerged with the use of immunological techniques (Lowenstein, 1980) currently used in parasite diagnosis with commercial kits (Gonçalves et al., 2002; Le Bailly et al., 2008).

The first report of ancient DNA and RNA extraction was presumably from 1980, by a group of Chinese researchers, based on rib cartilage from a mummy from Mawangtui, preserved for nearly 2,000 years (Herrmann & Hummel, 1994). However, Higuchi et al. (1984) published the first convincing demonstration of the recovery of aDNA sequences from an extinct animal. They showed the cloning of a 29 bp fragment of mitochondrial DNA (mtDNA) from a museum specimen of the quagga, a zebra-like species (*Equus quagga*) that became extinct in 1883. This huge study (25 thousand colonies analyzed for only two fragments of cloned *E. quagga* mtDNA) was a milestone in aDNA research, demonstrating for the first time the long survival of DNA after death (Chart 1).

Chart 1 – History of ancient DNA studies

Year	Study	Reference
1984	mtDNA from <i>Equus quagga</i> (1884)	Higuchi et al., 1984
1985	ntDNA from Egyptian mummy (2600 BC)	Pääbo, 1985
1989	Criteria for authentication of aDNA results	Pääbo, 1989
1990	cpDNA from Miocene <i>Magnolia</i>	Golemberg et al., 1990
1991	Determination of sex in 17 th -century human bones	Hummel & Hermann, 1990
1993	ntDNA from maize (4,700 BP)	Goloubinoff, Pääbo & Wilson, 1993
1994	Pre-Columbian tuberculosis	Salo et al., 1994
1997	Neanderthal mtDNA (not our direct ancestor).	Krings et al., 1997
1998	Giant sloth coprolite (11,000 BP): host and diet	Poinar et al., 1998
2000	16s rDNA from bacteria in Ötzi (5,530-5,100 BP)	Cano et al., 2000
2003	Paleoenvironmental analysis of permafrost	Willerslev et al., 2003
2006	Complete mtDNA genome of mammoth	Krause et al., 2006
2007	Detection of live ancestral bacteria in permafrost	Johnson et al., 2007
2008	Genotyping of <i>T. cruzi</i> in mummy (7,000-4,500 BP)	Lima et al., 2008

Shortly thereafter, the first results were published on human DNA recovered from an Egyptian mummy dated c. 2,600 BC. The study described cloning a 3400 bp fragment of nuclear DNA and the identification of repeat sequences in human DNA (Pääbo, 1985).

Previous aDNA studies were limited to DNA extraction from extraordinarily well-preserved material. However, technical difficulties in cloning DNA from ancestral remains were minimized by applying the polymerase chain reaction (PCR) (Saiki et al., 1985; Mullis & Fallona, 1987). With PCR, theoretically a single intact strand of DNA suffices to produce thousands of copies of the segment, making it an ideal tool for targeting aDNA.

The first decade of aDNA research witnessed an increase in interest by diverse groups and reports of aDNA recovery, exploring different types of preserved biological materials (Chart 2), mainly bones and tissues from mummies (Hagelberg et al., 1991; Merriwether; Rothhammer & Ferrell, 1994; Handt et al., 1996), insects in amber (De Salle et al., 1992; Cano et al., 1993), and fossilized plants (Golemberg et al., 1990; Brown et al., 1994).

Chart 2 – Ancient DNA in different biological materials

Origin	Type of material	Maximum recorded age (years)
Human	Bones and teeth	38,000
	Mummies	5,000
	Bog bodies	7,500
	Hair	0.3
Animal	Bones	25,000
	Naturally preserved tissues	13,000
	Tissues preserved in museum	140
	Plumage	130
	Fur	> 1
	Specimens in amber	135,000,000
	Permafrost	30,000
Bacterial	<i>Permafrost</i>	3,000,000
Viral	<i>Permafrost</i>	140,000
Fungal	<i>Permafrost</i>	750,000
Plant	Specimens in plant collections	118
	Seeds and cobs	4,500
	Mummified seeds and embryos	44,600
	Specimens in amber	40,000,000
	<i>Permafrost</i>	400,00

Source: Iñiguez et al. (2006).

From then on, numerous studies focused on standardizing PCR for studying aDNA, allowing the use of biological samples with degraded or minimal amounts of DNA. The studies featured the work by the group of Svante Pääbo, a leading figure in this research area from early on. His work in the extraction, enzyme amplification, cloning, and phylogenetic analysis of aDNA sequences revealed the peculiarities and problems of its manipulation and the possibility of obtaining proven ancestral gene sequences by applying various molecular techniques. Pääbo also pioneered aDNA characterization based on tissues from specimens representing a wide variety of geographic regions and periods, identifying the nature of chemical alterations in aDNA (Pääbo, Gifford & Wilson, 1988; Pääbo et al., 2004; Pääbo, 1989; Pääbo, Irwin & Wilson, 1990; Handt et al., 1994; Krings et al., 1997; Greenwood et al., 1999; Hofreiter et al., 2001, 2002; Hofreiter, 2008).

Ancient DNA research thus evolved and matured in the identification of problems and solutions. It moved from the mere amplification of DNA fragments from fossilized or extinct organisms to amplification of DNA from fossil material and the application of these studies to shed light on questions in other areas of knowledge. As of 1995, six extinct species had been analyzed phylogenetically. Currently, more than 20 extinct species of birds, humans, and other mammals have been elegantly studied and their mitochondrial genomes have been completed (Cooper et al., 2001; Poinar et al., 2006). One of the most relevant studies in human evolution was the mtDNA sequencing of Neanderthal, using sophisticated experiments to show that this species was not the direct ancestor of the modern human species as previously believed (Krings et al., 1997).

Finally, the scientific community showed growing interest in analyzing bacterial aDNA (Marota & Rollo, 2002; Cano et al., 2000; Rollo et al., 2000) from different human parasites such as *Mycobacterium tuberculosis*, *Yersinia pestis*, *Trypanosoma cruzi*, and mainly coprolites as a rich source of information on behavior, eating habits, and

parasites of ancient animals and humans (Cooper & Poinar, 2000; Hofreiter et al., 2001; Poinar et al., 1998, 2001; Loreille et al., 2001; Iñiguez et al., 2003, 2006; Leles et al., 2008; Gilbert et al., 2008).

ANCIENT DNA: APPLICATIONS

Until recently the study of evolutionary genetics was based mainly to extrapolate information from living organisms to reconstruct the past, or to interpret morphological analyses of well-preserved fossil specimens. DNA recovery from animal, plant, and human remains provided a new tool for obtaining primary evidence of ancient genetic information on these organisms. Ancient DNA analysis is definitely an innovative, high-impact, and extremely useful field of investigation that has led to progress in various areas of science.

Ancient DNA studies also apply to parasitology in a broader sense. The investigation of aDNA has been particularly successful in paleontology (Höss, 1995; Greenwood et al., 1999; Poinar, 2002), archaeology (Burger et al., 2000; Relethford, 2001), forensic medicine (Goodwin, Linacre & Vanezis, 1999; Góes et al., 2002), and even agriculture (Brown, 2001; Willmann, 2001) (Chart 3).

Chart 3 – Ancient DNA in various fields of knowledge

Lines of research	Principal studies
Anthropology	Sex prevalence and determination of behaviors Determination of diet and eating habits Origin and evolution of the human species
Archaeology	Human migrations and peopling patterns Identification of sex in human populations
Agriculture	Origin and dispersal of cultivars Domestication of maize and potato species
Conservation	Phylogenetic relations in mammalian populations
Forensic sciences	Identification of murder and accident victims Identification of war victims
Paleontology	Taxonomy of extinct plants and animals
Bacteriology	Determination of ancestral strains
Zoology	Taxonomy of recently extinct animals
Medicine	Origin of infectious diseases, tuberculosis, plague, leprosy
Parasitology	Intestinal parasites, Chagas disease, viral disease

CHARACTERISTICS OF ANCIENT DNA

The DNA in living cells remains intact through continuous processes of DNA repair, while the catabolic enzymes are restricted to cell compartments. When an organism dies, its DNA begins to be degraded by endogenous nucleases, reducing the DNA molecule to mononucleotides. Organisms that feed on or degrade macromolecules, such as bacteria, fungi, and insects, also attack the DNA. But this action can be prevented by the destruction or inactivation of these

enzymes under special conditions of rapid desiccation, low temperature, and high salt concentration (Lindahl, 1993; Hofreiter et al., 2001). Even so, processes persist that slowly affect the DNA, such as oxidation, depurination, deamination, and others like hydrolysis, the products of which modify nitrogenous bases and sugar-phosphate, and/or destabilize and break the DNA molecule. Degradation into small fragments, generally 100-500bp, is the most obvious damage to aDNA (Pääbo et al., 2004).

We now know that the extent of degradation does not depend only on the antiquity of the environments and the conditions under which the different archaeological materials were collected. The conditions and time of preservation following excavation and collection are central to the process of aDNA degradation (Pruvost et al., 2007). In tissue aDNA, oxidation and even the direct and indirect effect of background radiation frequently cause oxidative alterations in pyrimidine bases (cytosine C and thymine T), generating a high proportion of residues (hydantoins) that can inhibit the action of DNA-polymerase and consequently PCR. Deamination, or spontaneous hydrolysis of cytosine and its homologue 5-methylcytosine to uracil (U) and thymine, is also frequently found in aDNA, causing erroneous insertions during PCR (Lindahl, 1993; Hofreiter, 2008).

Based on data from oxidative and hydrolytic modifications, Hofreiter et al. (2001) showed that aDNA can be reasonably recovered from specimens as old as 100,000 BP under ideal conditions of physiological salt concentrations, neutral pH, and temperatures around 15°C.

This line of research thus entails extreme technical difficulties, for example when working with tiny amounts of DNA or degraded DNA. Studies of material from temperate environments or permafrost have demonstrated the possibility of pushing the limits of antiquity in DNA recovery and of the size of aDNA sequences.

Genetic analysis of permafrost showed that plant and animal aDNA can be preserved for long periods of time, even in the absence of macrofossils (Willerslev et al., 2003; Lydolph et al., 2005; Willerslev et al., 2007). For example, slices of permafrost from Siberia dated to 400,000 to 10,000 BP contained aDNA from at least 19 different plant taxa, including the oldest authentic aDNA sequence identified to date (Willerslev et al., 2003). A highly fascinating study of permafrost material showed the long survival of bacteria (*Actinobacteria*) in a detailed experiment that demonstrated metabolic activity and DNA repair in ancient microbial cells (Johnson et al., 2007).

AUTENTICATION OF RESULTS IN ANCIENT DNA

Current aDNA studies entail some certainties and many doubts (Marota & Rollo, 2002). In the mid-1990s, when several relevant aDNA studies proved to be irreproducible, the need to authenticate aDNA became evident. In addition, the recovery of DNA from specimens dated to more than 100 million years and the apparent identification of long aDNA sequences indicated contamination with modern DNA.

Unfortunately, it is now known that reports of false DNA have occurred ever since this line of research began. The first cloning of human aDNA from a fragment of approximately 3 kb (Pääbo, 1985) and the recovery of 0.8 kb of DNA from *Magnolia* chloroplast dated 17-20 million years (Golemberg et al., 1990) later proved to have resulted from contamination with modern DNA.

Likewise, reexamination of the pioneering study of the *quagga* specimen identified incorrect positions in the aDNA sequence in the original study. Some criteria for aDNA authenticity have been defined by the main research groups in this area and by specialists in nucleic acid biochemistry, thereby leading to greater rigor in publishing aDNA studies.

Pääbo (1989) provided the first definition of authenticity in amplified DNA. He basically highlighted the use of extraction controls, the detection of a strong inversely proportional correlation between efficiency of amplification and size of the amplified fragment, and reproduction of the aDNA sequence obtained in a second extract from the same sample. Subsequent studies defined steps and conditions for obtaining truly ancient DNA. They also suggested a series of control tests and techniques to be used in molecular experiments. The following is a description of various criteria for aDNA authenticity defined by the main research groups. For more details, see Cooper & Poinar (2000) and Pääbo et al. (2004).

Biochemical assays in macromolecular preservation

Since preservation of amino acids is consistent with that of DNA, biochemical assays in aDNA samples allow accessing the material's state of preservation and thus the classification of samples with possible DNA recovery. The most widely used method is the analysis of the amount, composition, and racemization of amino acids, used especially in bones and teeth, but also applied to coprolites. Poinar et al. (1996) were the first to demonstrate that specimens with high levels of amino acid racemization do not contain sufficient aDNA for analyses. Other methods include pyrolysis-gas chromatography, mass spectrophotometry, histological analysis, and transmission electron microscopy. However, we still lack extensive studies on the correlation between macromolecular preservation detected by these techniques and preservation of aDNA (Pääbo et al., 2004).

Physically isolated working area for aDNA

Before performing PCR, it is essential that work with aDNA be done in areas or laboratories restricted to this purpose. Physical and/or temporal segregation of the pre-PCR and post-PCR stages is essential to reduce the risks of exogenous contamination. It is obviously inappropriate to use a laboratory setting that routinely works with large amounts of DNA from the study's target.

Extraction and amplification controls

DNA extraction and PCR controls must be done in parallel for each experiment. The extraction control (in which the sample is replaced with autoclaved MilliQ or bidistilled H₂O) detects contamination of the procedure, reagents, and extraction facilities. When contamination is detected, work flows should be redesigned, including chemical and physical sterilization and disposal of reagents. PCR controls detect contamination of the PCR hood and primer. Positive controls are not recommended, and when necessary the amplification should be done outside the environment of the target aDNA amplification.

Quantification of molecules

The number of aDNA molecules in the extract should be determined whenever possible. This should be standardized for each set of primers, since primers differ in terms of efficiency in initial amplification. If this procedure shows a high number of molecules (> 1,000 molecules has been established as a good basis), multiple amplification or cloning will not be necessary. It is unlikely that changes in the sequence will occur, especially if a single target is expected (e.g., bone marrow extract – amplification of human mtDNA). However, with a small number of molecules (< 1,000 molecules) at the start of PCR, it is nearly impossible to rule out contamination, especially in human aDNA studies. In

this case the study requires new amplification, cloning, and sequencing with at least three clones to authenticate the result. The most widely used methods are competitive PCR and, more recently, real-time quantitative PCR.

APPROPRIATE MOLECULAR APPROACHES

As mentioned, an inverse relationship between the size and intensity of the PCR product is characteristic of aDNA. The recovery of 500 to 1,000 bp fragments is uncommon, and small fragments (<500 bp) are generally considered original aDNA. Likewise, if fragments are not more easily amplified than larger fragments when compared to modern DNA, this strongly suggests contamination. In analyses of simple copy targets (e.g., nDNA) or pathogens, reproducible amplifications of the host should be demonstrated, as in the detection of *Enterobius vermicularis* and human mtDNA in coprolites (Iñiguez et al., 2006). Nested PCR and reconstructive polymerization (Golemberg, Bickel & Weihs, 1996) are recommended.

The aDNA sequences should make phylogenetic sense, especially in studies of aDNA from extinct animals. When the small size of the aDNA fragment does not indicate it for this purpose, it is recommended to use overlapping sequences from small fragments, with confirmation of the sequences in the overlapping position.

The analysis of various overlapping fragments of aDNA provided the first analysis of Neanderthal DNA (Krings et al., 1997) and determination of complete mitochondrial genomes, as in the case of the mtDNA genome from extinct animals like the moa and mammoth (Cooper et al., 2001; Krause et al., 2006).

REPRODUCTION OF RESULTS

Ideally, a positive result of amplification is repeated in the same way and from other independent extractions of the specimen to verify sequence errors caused by aDNA damage. The result of aDNA sequences obtained from direct sequencing should be based on sequence analysis of 3 to 10 products cloned by amplification per sample, as mentioned.

INDEPENDENT REPLICATION

New, ambiguous, or unexpected aDNA results should be replicated in a second laboratory, specifically for human aDNA research. The work should be done from the aDNA extraction stage to the determination of the nucleotide sequence, preferably following the same experimental design and protocols. This eliminates the possibility of intra-laboratory contamination. Ideally, a subsample is sent directly from the museum or archaeological site to the second laboratory.

Nevertheless, studies are still published that fail to follow the criteria for aDNA research (Zischler et al., 1995; Monsalve et al., 1996; Nickle et al., 2002). This may be due to disagreement as to the applicability of the proposed criteria to a given study, as in biochemical assays of macromolecular preservation, possibly unnecessary when working with samples with visible preservation, recent dating, and adequate preservation after excavation. Adherence to such criteria in routine laboratory practice also makes the work expensive and extremely painstaking. For example, reproduction of results is not always easy, due both to the high cost of the experiments and the lack of specialized laboratories and professionals capable of reproducing the analyses (Cooper & Poinar, 2000; Montiel, Malgosa & Francalacci, 2001). It also appears practically impossible to totally eliminate the risk of contamination. Contamination has been detected, described, and discussed in the most highly specialized laboratories in the area (Handt et al., 1996; Kring et al., 1997; Marota & Rollo, 2002).

MOLECULAR PALEOPARASITOLOGY: BACKGROUND AND APPROACHES

The diseases that affected past civilizations and their etiological agents can also be studied by recovering original genetic material from the pathogenic agents.

One of the first such studies detected *Mycobacterium tuberculosis* aDNA in bones and discussed the importance of PCR for osteoarchaeological research (Spigelman & Lemma, 1993). The same methodology allowed identifying *M. tuberculosis* in lung tissue from a Peruvian mummy approximately 1,000 years old (Salo et al., 1994). Arriaza et al. (1995) later discussed the pre-Columbian origin of tuberculosis in Chile, presenting molecular evidence of the microbe in vertebral lesions. *M. tuberculosis* was also successfully amplified in vertebrae from the ossuary of an indigenous community in Canada (1400 AD) and an indigenous cemetery in Mississippi (1040 AD). These studies prove that tuberculosis was not introduced into the New World by the European explorers and colonizers, as previously believed.

The presence of tuberculosis in the Old World during pre-contact times was confirmed by identification of *M. tuberculosis* in a calcified structure in lung tissue from a body found in a Byzantine chapel dated 600 AD (Donoghue et al., 1998). To date, *M. tuberculosis* is the most widely explored microorganism in aDNA studies, not limited to diagnosis of the *M. tuberculosis* complex, but extending to genotyping of *M. tuberculosis* or *M. bovis* isolates and making important contributions to understanding the history of this disease (Taylor, 1996; Donoghue et al., 1998; Zink et al., 2001a; Fletcher et al., 2003; Zink, Grabner & Nerlich, 2005; Taylor et al., 2007).

Ancient DNA studies have been conducted to verify *Yersinia pestis* in the plague epidemics in Europe. Drancourt et al. (1998) were the first to demonstrate the bacteria in ancient human skeletons using molecular detection of *Y. pestis* in teeth from individuals dated to two epidemic periods (1590 and 1722). However, an analysis of 108 teeth collected from five archaeological sites potentially related to the plague in three European countries failed to yield positive results (Gilbert et al., 2004).

Different molecular targets have been used to attempt to explain discrepancies in results between aDNA studies (Drancourt et al., 1998, 2004; Raoult et al., 2000). Another question elucidated by aDNA studies was the determination of the genotype in *Y. pestis* isolates in ancient human remains from the same period in France and Germany during the plague pandemic. Multiple spacer typing (MST) and characterization of the *glpD* gene allowed concluding that the Orientalis biotype, now distributed worldwide, was the cause of the last epidemics in Europe (Drancourt et al., 2004, 2007).

The recovery of aDNA from bacteria and protozoa has also provided information on a variety of other infectious diseases such as leprosy (Rafi et al., 1994; Montiel et al., 2003), syphilis (Kolman et al., 1999), typhoid fever (Papagrigorakis et al., 2006), diphtheria (Zink et al., 2001b), gastrointestinal infection (Zink et al., 2000), and malaria (Nerlich et al., 2008).

By applying a molecular technique originally designed to detect *T. cruzi* in tissues of experimentally desiccated mice (Bastos et al., 1996), Felipe Guhl et al. (1999) obtained *T. cruzi* aDNA from tissues of mummies from the Atacama Desert, thereby concluding that 4,000 years ago, the prehistoric populations of southern Peru and northern Chile suffered from American trypanosomiasis (Chagas disease). The presence of pre-Columbian *T. cruzi* was also confirmed in the Atacama Desert of Chile, in mummies dated to 2,000 BP (Ferreira et al., 2000). A study established the diagnostic methodology based on hybridization of 70 bp PCR products from the minicircle region and showed that 25 of 27 various tissues from four Andean mummies were positive for this target (Madden et al., 2001). This approach later served to determine the prevalence rate of infection (41%) in populations from northern Chile to southern Peru, concluding that American trypanosomiasis has been present in the Americas for 9,000 years (Aufderheide et al., 2004).

A pioneering study in Brazil recovered a mini-exon fragment of *T. cruzi* aDNA from the rib bone of a female mummy dated 7,000-4,500 BP from the Peruaçu Valley in Minas Gerais State. This landmark study was the first to identify an ancient isolate as genotype *T. cruzi* I, suggesting that changes have occurred in the spatial and temporal distribution of Chagas disease, since Minas Gerais currently displays genotype *T. cruzi* II (Lima et al., 2008).

Finally, Fernandes et al. (2008) showed for the first time the link between *T. cruzi* aDNA genotyping and Chagas disease, studying a mummy with megacolon, suggestive of the disease.

Bacterial aDNA results were obtained from tissues of the Tyrolean Iceman Ötzi, dated to $4,546 \pm 17$ BP in the Neolithic period. The bacterial flora and pathogenic bacteria were identified by amplification of 16S ribosomal DNA obtained from tissue fragments of the stomach, colon, clothing, and others (Cano et al., 2000; Rollo et al., 2000).

However, this approach had to be carefully adjusted for aDNA studies, since we now know that 16S rDNA, widely used in bacterial taxonomy, only allows determination to the genus level, which could shed doubt on paleomicrobiological diagnosis.

Further interesting information was recovered on Ötzi by aDNA analyses, including his human mtDNA haplogroup (Rollo et al., 2006) and the content of his last meal before death (Rollo et al., 2002). The use of generic targets such as ribosomal RNA genes 12S and 18S and chloroplast DNA (cpDNA), which can determine the diet's plant origin, has been corroborated by phylogenetic analyses that provide taxonomic support for the diagnosis (Rollo et al., 2002). This work's main revelation was discerning between the origin of the plant aDNA from the gut content and that of the environment, i.e., diet versus pollen inhalation, using different molecular targets.

MOLECULAR PALEOPARASITOLOGY AND COPROLITES

The application of molecular techniques to the analysis of fecal material has served to produce individual and population information on diet, genus, filiation, and presence of pathogenic agents (Höss et al., 1992; Kohn et al., 1995, 1999; Kohn & Wayne, 1997).

Fricker, Spigelman & Fricker (1997) used PCR to detect *Escherichia coli* aDNA in the intestinal content of Iron Age mummies, dated 300 BC. The study revealed several inherent methodological difficulties in working with feces (inhibition of enzyme amplification) and DNA (degradation of the aDNA and contamination with modern DNA). The authors also highlighted how coprolites can serve as a source for analyzing infectious diseases in prehistoric civilizations, besides providing alternative information on the evolution of these pathogens.

Leading aDNA experts failed in their initial attempts to recover DNA from coprolites (Höss et al., 1996). However, Vasan et al. (1996) demonstrated the presence of large amounts of ligand structures between groups of amino acids and reduced sugar (Maillard products) in coprolites and showed that phenacylthiazolium bromide (PTB) cleaves this structure, thereby facilitating aDNA amplification.

Thus, it was not until 1998 that aDNA from animal coprolites nearly 19,000 years old were analyzed to determine their origin and the composition of the animals' diet (Poinar et al., 1998). The technique was amplification of ribosomal genes from the chloroplast and mitochondrial genes and subsequent species identification by nucleotide sequence analysis of various clones. Poinar et al. (2001) also demonstrated the diversity of Amerindian diet using three coprolite samples more than 2,000 years old.

However, despite its rigorous methodology, the latter study was flawed in pointing to species in the plant diet since it failed to consider the region's macromolecular findings and palynological studies (Reinhard et al., 2008). The

latter authors emphasize two basic critiques of the study: 1) the small cpDNA fragment that was analyzed would only allow taxonomic classification to the family level and 2) the approach for species identification was simple selection of the highest percentage of similarity between sequences after comparison with the international nucleotide sequence databank (Blast/GenBank), where data on the cpDNA sequence that correspond to the species from the Amerindian plant diet may not have been studied or deposited.

Coprolites are mainly used as a source for paleoparasitological research. Loreille et al. (2001) recovered aDNA from *Ascaris* eggs isolated from medieval coprolites. This detailed study used citB and 16S genes as the target and performed comparisons with DNA sequences from modern *Ascaris lumbricoides* and *Ascaris suum*, parasites of humans and swine, respectively. Although the archaeological analysis did not determine the origin of the coprolites (human origin was only suggested), it is now known that cross-infection exists and that species classification is much more complex. At any rate, confirmation of the presence of *Ascaris* sp. in 14th-century coprolites recovered from a latrine at the Place d'Armes archaeological site in Namur, Belgium, was the first encouraging molecular evidence of nematode enteroparasites in ancient European populations.

Iñiguez et al. (2003, 2006) studied pre-Columbian coprolites from four archaeological sites in North and South America, dated to 4110 BC-900 AD. They recovered *E. vermicularis* aDNA using the 5S rRNA spacer region as the molecular target. The study was the first to show the possibility of accessing genetic material from the parasite even without prior isolation of eggs and in samples that were negative on paleoparasitological analysis. It featured a genetic analysis of the intergenic region that proved to be highly conserved temporally and spatially, due to the presence of an inserted gene (SL1 RNA) that imprinted conservation on the target region (Iñiguez et al., 2006).

Direct molecular paleoparasitological diagnosis of coprolites and sediments has shown positive results in samples that were negative for *Ascaris* sp. under light microscopy. Unlike microscopic analyses, molecular techniques based on the method proposed by Loreille et al. (2001) showed that *Ascaris* and *Trichuris* were widely distributed in pre-Columbian South America and that the distribution pattern of these infections at the time did not differ greatly from the current pattern (Leles et al., 2008). Molecular diagnosis has proven more sensitive than light microscopy for diagnosing *Ascaris* sp and *E. vermicularis* and can be expected to make important contributions to the evolutionary history of these parasites. It should thus be extended to the study of other parasitic infections.

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