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

18. Paleoparasitology and Ancient DNA

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Paleoparasitology and Ancient DNA

Katharina Dittmar

Insights into the presence and composition of parasite fauna through time can be obtained either by direct specimen evidence from fossil or archaeological material or by identifying or sequencing parasite DNA from the samples.

Direct evidence prevailed in the early days of paleoparasitology and was advanced by the development of coprolite rehydration techniques (Callen & Cameron, 1960) and the modification and refinement of regular parasitological diagnostic techniques for paleoparasitological purposes (Reinhard, et al., 1986; Reinhard, 1988; Reinhard, 1992).

Evidence from direct methods mainly concerns parasite eggs and protozoan cysts. However, issues of taxonomic resolution often limit these techniques, since many parasite remains cannot be identified to the genus or species level.

The development of ancient DNA techniques allowed tracing parasitic diseases at the molecular level, sometimes without direct clues of parasitic infection or disease. Sequencing of parasite DNA began with *Trypanosoma cruzi* DNA from Chilean mummies (Guhl et al., 1999). Reports now cover a wide range of parasites, including protozoans, helminthes, and arthropod ectoparasites. This chapter reviews paleoparasitological DNA studies in the context of phylogenies, population genetics, and phylogeography.

SAMPLES AND ANCIENT DNA PRESERVATION

Ancient DNA pertains to specimens that range from a few decades old (e.g., museum specimens) to roughly 100,000 years (Knapp et al., 2008). According to Pääbo et al. (2004), any DNA reported to be millions of years old is actually an artifact.

The most conspicuous materials used in paleoparasitological research are coprolites and latrine sediments. Only occasionally, ectoparasites, soft tissues (e.g., from mummies), and bones are available. In mummy studies, the parasite's life cycle should be considered in order to choose the tissue that most likely contains developmental stages or DNA. An example is intestinal or heart tissue for *Trypanosoma cruzi*. In this case, crushed bone can also be used

in the search for vestiges of preserved bone marrow, since *T. cruzi* reproduces in hematopoietic tissue (there is a high likelihood of recovering its genetic material from various tissues, especially those that produce or accumulate blood).

Most currently used paleoparasitological evidence concentrates on human samples (Horne, 1985). However, tremendous potential exists to study parasitism of animals, both domesticated and wild. Sources of animal paleoparasitological evidence include animal mummies (Dittmar, 2000; Shapiro & Cooper, 2003; Beckman, 2004; Knudson et al., 2006). Other samples pertain to animal burials or preserved animal enclosures (Jones et al., 1988; Hall & Kenward, 1998; Nielsen et al., 2000; Dittmar & Teegen, 2003). Most of these examples are from domesticated or semi-domesticated animals. However, material from wild animals includes fecal materials accumulated over long periods of time in protected environments (such as permafrost or caves), like packrat or caribou middens, owl pellets, or bat guano, holding potentially important parasitological clues (Betancourt et al., 1990; Scott et al., 1996; Bell, 2000; Kuch et al., 2002; Smith & Betancourt, 2006; Leroy & Simms, 2006; Maher, 2006).

Several palynological and dietary studies have also been performed on hyena coprolites (Horwitz & Goldberg, 1989; Carrión et al., 2000; Carrión et al., 2001; Yll et al., 2006).

Depending on the age and state of preservation, these samples may still contain DNA in varying stages of degradation. Specifically, this means that DNA is fragmented (100-500bp) and only present in low concentrations (Knapp et al., 2004). Additionally, mitochondrial DNA is preserved better than nuclear DNA, presumably due to endogenous differences in copy number (Pääbo et al., 2004). For instance, analysis of a 700 year-old Amerindian cemetery found that only 15% of the samples yielded single-copy nuclear DNA (Stone & Stoneking, 1999).

Several methods have been developed to exclude samples that are unlikely to yield any results (Knapp et al., 2008). One approach assesses the biochemical preservation of a sample through amino acid conservation and concentrates on the evaluation of aspartic acid levels in the sample (Poinar & Stanckiewicz, 1999; Pääbo et al., 2004). If a significant reduction beyond a certain level occurred, the sample may not contain amplifiable DNA. However, higher levels do not guarantee that DNA is preserved (Poinar & Stanckiewicz, 1999; Kolman & Tuross, 2000; Burger et al., 2007). This approach is also limited by the available sample size and is not recommended for coprolites. Among paleoparasitological samples, Dittmar et al. (2003) used this test to assess the possibility of DNA amplification from a mummified flea (*Pulex irritans*).

Another test uses competitive or real time PCR to assess the amount of starting template. According to current experience, with less than 1,000 template molecules, recovery of aDNA is highly unlikely (Pääbo et al., 2004). Specific damage to DNA molecules includes: strand breaks, DNA crosslinks, hydrolytic damage, and oxidative lesions (Pääbo et al., 2004). Therefore, strict adherence to established protocols is necessary, an issue reviewed elsewhere (Austin et al., 1997; Yang, 1997; Wayne et al., 1999; Cooper & Poinar, 2000; Pääbo et al., 2004; Willerslev & Cooper, 2005).

COLLECTION AND STORAGE OF MATERIAL

As with any other organic material, care should be taken when collecting paleoparasitological samples and storage conditions should be optimized to avoid further degradation of the material. Use of gloves is recommended while handling specimens. To avoid premature and uncontrolled rehydration, coprolites should be stored under dry conditions at cool or freezing temperatures.

When working with latrine sediments, control samples from the adjacent (non-latrine) environment should be taken to establish a comparative baseline sample. With latrine sediments, the possibility of recent parasite contamination

should also be taken into account. This is especially important when settlement sites were continuously occupied through long timeframes, and contamination of older layers from younger deposits is possible. Arthropod remains (e.g., lice, ticks, fleas, or mites) should be stored according to standard entomological techniques (Bouchet et al., 2003).

ANCIENT DNA TECHNIQUES

Generally, aDNA techniques vary little from standard modern DNA studies. However, usually very little aDNA starting template is available, additionally complicated by the fact that aDNA is fragmented into short pieces (see above). When working on human aDNA, surface decontamination has been applied to samples (e.g., mechanical removal of the surface; Knapp et al., 2008), but this is not always effective (Kolman & Tuross, 2000).

Similar techniques using surface UV light exposure have been applied to coprolite studies (Iñiguez et al., 2003a) and are warranted if one suspects surface contamination with closely related extant parasite species or DNA. In fact, little is known about the possibility of water percolation and particle or DNA movement through sediment layers (Pääbo et al., 2004), and systematic studies to this effect are currently lacking.

Methods for the extraction of ancient DNA depend on the materials used. If bone samples are involved, a decalcification step is recommended. Most historical or ancient samples are also known to contain substances that potentially inhibit polymerase chain reactions (PCR). Membrane filtration or resin binding techniques work best for cleaning the extracted DNA (Roland & Hofreiter, 2007).

Research on coprolites has identified the presence of Maillard products, which are the result of reactions between sugars and primary amino groups and hinder the activity of polymerase (Pääbo et al., 2004). The reagent N-phenacylthiazolium bromide (Vasan et al., 1996) was successfully used on coprolites (Poinar et al., 1998) and Neanderthal bones (Krings et al., 2000) to break down Maillard products and allow DNA amplification.

Ancient DNA amplification employs the regular steps of denaturation, annealing, and extension, but multiple positive and negative controls are applied to check for contamination. In addition, all reactions should always be independently replicated by different researchers in other laboratories, or the results will remain doubtful.

The recent development of multiplex DNA techniques provides the opportunity to increase the length of obtainable sequence fragments from limited amounts of starting template (Krause et al., 2006; mitochondrial genome of Pleistocene woolly mammoth).

Multiplex DNA uses multiple primer pairs in one PCR reaction to target subsequences within the total DNA sample. In a follow-up simplex PCR the regions of interest can then be sequenced from an already multiplied DNA fragment pool (Krause et al., 2006; Römpler et al., 2006).

Another recent technological advance is ancient genomics, where the total DNA of a sample is amplified (rather than individual sequences) (Lambert & Millar, 2006). The success of these methods can be seen in recent reports on the ability to generate genome data from ancient material (Noonan et al., 2006; Green et al., 2006).

Contrary to the previously mentioned multiplex approach, these techniques amplify the target DNA, together with all other DNA, including contaminants. The idea of analyzing “bulk” samples is not new and has been described as a metagenomics approach for environmental samples (Handelsman, 2004; Venter et al., 2004).

A typical metagenomics project starts with the construction of a bacterial clone library from random DNA of different origins from an environmental sample.

For the often scarce ancient DNA in samples, cloning provides the advantage of enhancing the total number of available template copies. However, statistically speaking, rare “ancient” inserts will still only provide a minimal proportion of all obtained clones, since most contain the more common DNA template copies (contaminants). Therefore, many clones have to be sequenced in order to identify the insert of interest.

This approach was used initially by Noonan et al. (2005) to sequence Neanderthal DNA. In order to save time and resources in sequencing the more abundant contaminant DNA, they also developed a new approach using biotinylated sequence fragment probes devised from known human DNA to “highlight” particular clones of interest, which were then selectively sequenced.

This approach works well in paleoparasitological samples if one is interested in obtaining the sequence of a particular parasite in a sample. In the case of typing the entire parasitological DNA in a coprolite, latrine sediments, or mummy tissue, the environmental shotgun sequencing (ESS) approach should be performed (Venter et al., 2004; Eisen, 2007).

To obtain a comprehensive overview of all preserved parasite DNA in a sample, statistically representative sequencing of many clones is necessary. This has become more feasible with the onset of a high throughput technique called “pyrosequencing” (Margulies et al., 2006). Green et al. (2006) used pyrosequencing to perform direct large-scale sequencing of Neanderthal DNA. In this approach, single-stranded DNA fragments were paired with common adapters and amplified by bead-based emulsion PCR (solid-phase pyrosequencing). Each bead contains several million copies of DNA fragments, which are then sequenced [GS20 454 sequencing system].

The advantage of this method is that it avoids bacterial cloning (common in the traditional metagenomics approach) and therefore prevents the loss of copies during transformation in the vector and subsequent growth of the colonies (Green et al., 2006). The approach generates thousands of sequence reads per run, thus increasing the odds of sequencing aDNA over contaminants (Green et al., 2006; Stiller et al., 2006). Additionally, patterns of artificial substitutions (due to misincorporations in the sequencing process) can be used to assess the probability of the resulting sequence being authentic. This is not possible with traditional Sanger sequencing, since it uses double-stranded DNA, where one does not know the template strand from which the final sequence (single-stranded) was generated (Stiller et al., 2006).

Currently, aDNA evidence exists for a variety of parasites, including *Trypanosoma cruzi* (Guhl et al., 1999; Madden et al., 2001; Aufderheide et al. 2004, Lima et al., 2008), *Leishmania* sp. (Zink et al., 2006), parasitic helminths (Loreille et al., 2001; Iñiguez et al., 2003a, Iñiguez et al., 2003b, Leles et al., 2008), and ectoparasites (Dittmar et al., 2003; Raoult et al., 2006; Raoult et al., 2008). None of these studies uses a genomics approach, but all are based on sequencing individual genes.

When working with extant parasite DNA, sometimes RFLP (Restriction Fragment Length Polymorphism) or microsatellite data are used, especially in population studies. However, these approaches are not recommended for paleoparasitological population studies. Due to the degraded nature of the template DNA, nucleotide misincorporations (errors) during amplification are possible, and are unlikely to be detected with RFLP or microsatellite analyses (Knapp et al., 2008).

In some cases, ELISA (enzyme-linked immunosorbent assay) techniques have been used to trace a parasite in paleoparasitological samples. ELISA techniques detect the presence of proteins (i.e., enzymes, surface proteins of viruses or bacteria, etc.). Therefore, the approach is only suitable for detecting a parasite, and does not allow for evolutionary analyses (i.e. phylogenies).

For instance, Deelder et al. (1999) employed ELISA techniques to detect parasite antigens in mummy remains from Egypt, to verify the presence of *Schistosoma* sp.. Mitchell et al. (2008) recently used ELISA to study for dysentery in two medieval latrines in the city of Acre.

However, as with DNA, protein degradation is a progressive and consistent phenomenon after death. Therefore, multiple negative and positive controls should be applied to check for false positives and potential cross-reactions.

EVOLUTIONARY ANALYSES

Molecular evidence from paleoparasitological samples can provide insight into the evolutionary history of parasites and diversity of parasite populations over time. Crucial to the endeavor of analyzing ancient DNA is the fragment assembly and identification of the obtained sequences.

For mixed sequences from multiple species (i.e., from metagenomic approaches), analysis is usually performed by putting homologous sequences together with a comparative assembler, or a program that can deal with draft sequence.

Basic local alignment search tool (BLAST) searches (e.g., GenBank) and subsequent analysis of all homologous sequences by phylogenetic approaches then allow for the identification of the obtained sequences. However, identification is often only possible at higher taxonomic levels, and genus and species identification is only feasible if similar or identical sequences are available for the gene of interest.

Therefore, in genomic approaches, a majority of recovered DNA may remain unidentifiable for the time being (Green et al., 2006; Noonan et al., 2006). If a particular parasite species is targeted in a paleoparasitological sample, it is recommended to search for genes that have known extant representation in GenBank. Genes or regions that are known to be informative for the question at hand should be sequenced, as derived from alignments with extant sequences (Dittmar et al., 2007).

The relevant current methods of phylogenetic analysis are maximum parsimony, distance methods, maximum likelihood, and Bayesian inference. Ideally, the inference method used will extract the maximum amount of information available in the sequence data. Phylogenetic analyses mainly aim to infer the evolutionary history of a group of species, or groups of genes (i.e., gene family evolution). When working with aDNA, such analyses often serve the purpose of sequence validation and identification. In other words, the fact that aDNA sequences group with homologous extant genes on a phylogeny identifies the sequenced piece as closely related and thus allows the researcher to assess whether the aDNA belongs to the species (or taxonomic group) of interest.

Because aDNA samples are generally not older than ca. 100,000 years, many genes will lack an accumulation of informative variation to distinguish them from their extant counterparts (in other words, they are too closely related). This problem is aggravated by the fact that often only certain genes are obtainable, which may not be suitable for the question asked and may prevent phylogeny estimation from multiple independent loci (Pääbo et al., 2004). For instance, the study by Raoult et al. (2008) on pre-Conquistador head lice (*Pediculus humanus capitis*) from Peruvian mummies employed two mitochondrial genes, cytochrome B and cytochrome oxidase subunit I. This provided enough information to cluster the sequenced lice with the worldwide haplotype (phylotype A) in a phylogeny derived from extant louse data.

While this allowed rejection of the long-held hypothesis of head louse introduction into the Americas by the Spanish Conquista and supports the hypothesis that body lice may also have been present in the pre-Hispanic Americas, it did

not support further interpretation regarding the specific geographic origin of South American lice by an earlier human migration, due to lack of resolution at the intra-population level.

Similarly, the study by Dittmar et al. (2003) on pre-Hispanic *Pulex irritans* (human fleas) clearly showed grouping of the historical sample with extant *Pulex* from the same geographic area, but the obtained genes did not show intraspecific variation.

In the paleoparasitological realm, the phylogenetic study of higher taxonomic levels (families, orders) and the relationship of extinct to extant species are difficult, simply because the preservation of parasites through time is very rare. Soft-bodied endoparasites are especially unlikely to be preserved.

Furthermore, parasites tend to occupy niches on their hosts that do not readily lend themselves to preservation (e.g., fur, feathers, digestive tract, soft tissue). Consequently, parasites are certainly underrepresented in the fossil record, which poses a continuous problem for the calibration of divergence time studies on parasite phylogenies. The occasional evidence of fossil parasites mainly concerns arthropods (Poinar & Poinar, 2007).

Other fossil data include amber-preserved nematode parasites (Mermithidae) from Neotropical ants (Poinar et al., 2006) and trace evidence from polychaete borings in pelecypod, gastropod, cephalopod, coral, stromatoporoid, crinoid, brachiopod, ectoproct, and calcareous algal skeletons from the Ordovician to the Permian, and from the Paleozoic (Cameron, 1969). However, none of the above is likely to yield remnants of DNA.

Currently underexplored avenues of DNA-grade parasitological evidence are permafrozen mammoth remains (importantly, the odds of their discovery are likely to increase with rising annual temperatures). Several studies on genetic data from mammoths and mastodons (Cooper et al., 2001; Poinar et al., 2005) and their dietary remains (Hofreiter et al., 2000) have proven the feasibility of DNA recovery from this material, and parasite eggs may also be preserved.

An example for the successful phylogenetic study of extinct remains comes from the sequencing of the mitochondrial ND2 and cytochrome B genes of the giant Haast's eagle (*Harpagornis moorei*), which not only showed an unexpected relationship to a group of small eagles but also supported a rapid morphological change over a small evolutionary time frame (0.7 – 1.8 my) (Bunce et al., 2005). If carried out on paleoparasitological material, results may similarly be informative on parasite divergence times and plasticity of morphological adaptation.

Another promising area with ancient parasite DNA is the study of animal domestication, or in a broader sense, the cultural association between humans and animals (e.g., rats, mice). Domestication not only increases the number of available hosts in a defined habitat, but also fosters contact between animal parasites and human hosts, and vice versa (Araújo et al., 2000; Cox, 2002; Kloos & David, 2002). Domestication thus provides parasites with a relatively stable number of readily available hosts and expands the potential to find new hosts. Familiar examples include many secondary human parasites, which are in fact primary parasites of other animal hosts, such as *Trichinella* spp. (primarily found in rats and pigs), *Trypanosoma cruzi* (sylvatic and domesticated mammals), *Balantidium* spp. (pigs), or *Pulex irritans* (guinea pigs).

Domesticated animals are often suspected as the origin of human parasitoses. However, *Taenia* provides an example of how genetic evidence from extant samples suggests acquisition of the infection by human ancestors before domestication of the respective ungulate hosts (Hoberg et al., 2001). Therefore, paleoparasitological evidence can be informative on multiple fronts. First, it can help identify parasitic infections that are common to both animal and human samples by microscopic parasitological diagnostics, or genetic sampling. Second, in most cases, the paleoparasitological sample can be dated, that is, within the context of other evidence. It thus provides a time point

that can be used to calibrate molecular dating efforts, which is particularly valuable given the persistent lack of parasite fossils. This will subsequently aid determination of the original host and the direction of host switches.

Occasionally, aDNA studies center on the species or population level. Not all samples lend themselves to this type of analysis, since a statistically satisfactory sample is needed to effectively assess haplotype frequencies. For instance, Burger et al. (2007) studied two nuclear loci for lactase persistence from 10 Neolithic, Mesolithic, and Medieval individuals.

Lactase is necessary to digest lactose (milk sugar), and most mammals lose this ability after weaning. Only the Medieval individuals showed the required heterozygosity for lactase persistence. Therefore, this trait was probably only acquired by humans in the last 8,000 years, due to strong positive selection.

Crucial to this type of study is the comparative combination of extant and ancient (or historical) samples from the same geographic area. This provides for a comprehensive diachronic observation and may also allow assessing recent host switches in the case of paleoparasitological samples.

No comprehensive study of this type exists for paleoparasitological samples, although Loreille & Bouchet (2003) highlighted the possibility of assessing speciation patterns, introgression, and hybridization with *Ascaris* spp. samples from extant material and a 14th-century latrine from Belgium. The proposals by Loreille & Bouchet (2003) are interesting in the context of coalescent approaches, which seek to retrospectively trace extant alleles to their most common recent ancestors (MRCA) through time (Edwards & Beerli, 2000) and thus gain an understanding of genealogic and population history, including events of hybridization or introgression. Time points of coalescence are estimated from the extant data, and having a dated sampling point from a potentially extinct allelic type (from the paleoparasitological sample) will likely improve the model's accuracy.

However, closely related species (populations) often display shallow coalescence times, and thus many alleles will be impossible to distinguish between species (e.g., Neanderthals versus humans, Knapp et al., 2008). Therefore, this type of study is subject to limitations, depending on the available material and the ability to find and amplify the right markers.

PALEOPARASITOLOGY AND PHYLOGEOGRAPHY

Host and parasite evolution are intricately linked. Genetically and morphologically, they engage in a constant race, each trying to gain advantage over the other. These connections persist through time and space. In this process, parasites are passed on from parents to offspring, and from ancestors to descendants.

Together with their hosts, parasites move around, and in due time adapt to new ecosystems. Paleoparasitological research provides a glimpse into these past events and may thus inform us directly on host migration routes and host switches, or indirectly on climate conditions, host nutrition, and cultural practices (Reinhard & Bryant, 2008). This is important, because the study of extant populations alone can lead to misperceptions concerning temporal distribution patterns of parasites or their hosts.

For example, paleoparasitological data on hookworms, threadworms, and whipworms provide solid evidence of non-Beringian human travel routes into the New World, thus refueling an intense debate on the issue (Hawdon & Johnston, 1996; Sianto et al., 2005; Montenegro et al., 2006; Araújo et al., 2008). These studies particularly support the idea that in some cases the Arctic environment may have acted as a barrier to certain Old World diseases (Dillehay, 1991).

Araújo et al. (2008) argue that hookworms (*Necator* sp. and *Ancylostoma* sp.), whipworms (*Trichuris trichiura*), and other helminths require specific conditions to complete their life cycle, which were not met in the hostile Beringian climate. Specifically, they point out that human hookworm and whipworm, which are host-specific and lack intermediate hosts, favor high moisture and moderate temperatures for egg and larval development.

Combining the paleoparasitological data with migration models that consider parameters such as human dispersal, it is unlikely that humans crossed Beringia fast enough to introduce these parasites into the New World. Therefore, the Clovis migration may not have been the only route for human arrival, and coastal or transoceanic migrations are possible (Araújo et al., 1988; Montenegro et al., 2006).

Montenegro et al. (2006) draw on direct parasitological evidence in the form of parasite eggs. Direct evidence is equivalent to morphological (phenotypic) data, which often does not provide resolution at the population level. For instance, the study by Araújo et al. (2008) cannot determine from which Old World population of whipworms or hookworms their New World counterparts descended. Similarly, although Raoult et al. (2008) used molecular data, their genes did not provide the appropriate resolution to resolve the genealogic history of lice.

In order to study sequence variation among individuals from a given species, DNA data from appropriate genes are necessary. However, such studies are only feasible if samples from multiple individuals are available (rarely the case for paleoparasitological samples).

Studies on human samples included DNA from early Europeans to study the ancestry of modern Europeans (Haak et al., 2005). Haplotypes from 24 skeletons showed that the current European genetic composition likely derives from Paleolithic hunter-gatherers inhabiting the area some 40,000 years ago (Knapp et al., 2008).

Paleoparasitological data may have implications for our understanding of global warming and its influence on the transmission of parasitic diseases. Because most parasites require certain conditions for their successful development, temperature fluctuations may allow or drive parasites to migrate and colonize areas other than their extant distribution (see above, Araújo et al., 2008). Combining paleoparasitological and paleoclimatic data with information on extant climate and parasite distribution could thus improve the long-term predictive power regarding the parasitological consequences of climate change on the local and global geographic scales.

For instance, Lima et al. (2008) recently found DNA from *Trypanosoma cruzi* I genotype in human remains 4,500 to 7,000 years old from the Peruaçu Valley in Brazil. This genotype is currently absent from the area and indicates the recent emergence of a new epidemiological profile, due to shifts in host availability, which may (according to the authors) be linked to deforestation and microclimate shifts.

Additionally, most paleoparasitological samples also contain remnants or DNA sequences of plants, invertebrates, or vertebrates, thus allowing us to directly link parasite data to other fauna and flora at the spatial and temporal levels (Nielsen et al., 2000). Connecting these useful glimpses into the past to the present is important, since it can provide clues to a variety of processes, such as alternative transmission cycles, coevolution, feedback relationships between diet and infestation levels, and the impact of human activity on infestation cycles.

CONCLUSIONS

Technical advances in experimental and computational analyses continue to inspire the field of paleoparasitology. In particular, the ability to sequence ancient DNA has led to major advances in the field. However, a critical attitude towards aDNA results from paleoparasitological material is necessary, and above all, the limitations of aDNA research

have to be taken into account. These problems concern the sample's age and the conditions of its preservation, as well as contamination, sequencing artifacts, and computational analyses.

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