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Paleomicrobiology: a Snapshot of Ancient Microbes and Approaches to Forensic Microbiology

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Abstract

Paleomicrobiology, or the study of ancient microorganisms, has raised both fascination and skepticism for many years. While paleomicrobiology is not a recent field, the application of emerging techniques, such as DNA sequencing, is proving essential and has provided novel information regarding the evolution of viruses, antibiotic resistance, saprophytes, and pathogens, as well as ancient health and disease status, cultural customs, ethnic diets, and historical events. In this review, we highlight the importance of studying ancient microbial DNA, its contributions to current knowledge, and the role that forensic paleomicrobiology has played in deciphering historical enigmas. We also discuss the emerging techniques used to study the microbial composition of ancient samples as well as major concerns that accompany ancient DNA analyses.

WHY STUDY ANCIENT DNA?

For the first time, ancient and modern genetic research is going hand in hand (1).

Eske Willerslev

Nucleic acids and modern sequencing analyses are the pivot for research in microbiology, as they provide insights into microbial gene pools and essential cellular processes. Particularly, the ability to isolate and sequence ancient DNA (aDNA) is of increasing importance, as it demonstrates gene evolution and also allows us to peek into the possible mechanisms for DNA preservation. aDNA studies have raised both fascination and skepticism, yet information yielded by such studies has served as the basis to understand the origins and evolution of animal and human groups (2–4), culture (5, 6), diet (7), and ancient diseases (8).

The study of aDNA is not restricted to extinct animals and plants, as it applies to recoverable DNA from any life form preserved in ancient specimens, including microorganisms (9). Paleomicrobiology, or the study of these ancient microorganisms, may go back as far as the 1920s, when researchers, including Chas B. Lipman, isolated microbes from coal dating back millions of years. Lipman was also one of the first to state that dormancy, or a state of

“suspended animation,” may be reached by microbes, thus making their isolation possible after long periods (10, 11).

Lipman’s results were predicated on careful microbial isolation techniques as well as rigorous controls. However, paleomicrobiology was not truly recognized as a scientific field during the first half of the 20th century due to skepticism about the ability of microorganisms to survive thousands of years in preservation. This is mostly because it was thought that those microorganisms found in ancient artifacts were the result of modern contamination. As a result, paleomicrobiology has evolved into a field based on stringent aseptic measures and experimental controls, allowing it to grow in its scientific relevance (12). For example, permafrost, deep terrestrial and marine subsurfaces, and fecal masses and coprolites have been used to study entire ancient microbial communities, including ancient bacteria, archaea, eukaryotes, and viruses (13–17). Similarly, specimens, especially those of human origin, have been used by forensic paleomicrobiologists to unravel important ancient diseases that have afflicted humankind in the past, including parasites, viruses, and bacterial infections. Throughout this review we mainly focus on discoveries made by forensic paleomicrobiology; however, nonclinical sample studies are also discussed, as these have provided important insights into the evolution of life, antibiotic resistance, and metabolic processes within microbial communities, among many others.

MAJOR CONCERNS FOR aDNA STUDIES

Low DNA Yield and Modern Contaminants

One of the major concerns regarding aDNA studies, including that of microbial origin, is that the molecule itself is prone to rapid degradation due to hydrolysis and chemical damage (8, 18, 19), resulting in very low aDNA yield (20, 21). Common practices used to overcome this problem include DNA concentration methods using glycogen and alcohol precipitation, as well as incorporating a preamplification step before sequencing. However, these procedures may also increase the risk of contamination with modern DNA. For this reason, appropriate working conditions and postsequencing aDNA authentication are vital in paleomicrobiology (22). First, in order to eliminate any contaminating sources, aDNA isolation and studies must be carried out in an appropriate and exclusive area, with designated reagents and equipment that should not leave the premises. DNA studies have also been performed in industrial facilities earmarked exclusively for this type of analysis (23). In general, sample handling procedures and precautions used in aDNA studies should be similar to those used in forensic laboratories.

In the case of molecular anthropological studies, there is a particular need for additional precautions. For example, DNA from those handling the fossils should be obtained in order to screen for modern DNA contamination on the samples. However, such additional precautions are not compulsory when focusing exclusively on the microbiome preserved in these samples. Second, a variety of authentication controls are compulsory for the validation of results. For example, positive controls (when used) should not be stored or handled in areas or with equipment designated for aDNA.

The analyst should be aware that DNA degradation may have occurred over time and that this must be taken into consideration. As time passes, fragmentation of the DNA molecule will increase as a result of exposure to ionizing radiation and/or enzymatic processes; therefore, the average length of an ancient DNA sequence in a sample could be less than that of modern DNA contaminants (24). This expected degradation pattern can serve as validation of aDNA obtained from samples, although observed patterns may vary depending on preservation conditions. Results should also be reproducible by independent researchers. With the use of a strict protocol and controls, molecular paleomicrobiology is undoubtedly a powerful tool for the study of ancient samples (22, 25).

How Can DNA Be Detected after So Long?

The mere presence of good-quality aDNA preserved in archaeological samples has been cause for debate (26, 27). Given its theoretical degradation rate, the recovery of sufficient DNA for further applications is highly surprising and may raise the question of whether external sources of contamination are predominant in the sample (28, 29). The suggested half-life of eukaryote DNA in fossils is less than 600 years; however, studies have obtained aDNA from animal and human samples predating the present by more than 10,000 years (30–32). Environmental degradation of DNA is mediated by an array of enzymatic and nonenzymatic processes, among which are hydrolytic activity, glycosylation oxidation, and methylation events (33, 34). What is apparent, however, is that prokaryote DNA seems to be more resilient to degradation, as studies have detected microbial DNA dating back almost 400,000 years in environments optimal for long-term preservation (21, 26). Such environments are mainly characterized by an absence of available water molecules, as this slows enzyme-mediated processes such as depurination and thus maintains the stability of the molecule for longer periods. This does not, however, explain how DNA “survives” ionizing radiation.

In addition to water availability, several intrinsic characteristics of prokaryotes could account for a greater protection against degradation. It is generally believed that intracellular DNA preserved within dormant or dead microbes is better protected against degradation than naked DNA. Moreover, the composition of a microbe’s cellular membrane plays a key role in the postmortem preservation of its genome. For example, membranes rich in mycolic acids, such as those in mycobacteria, are less likely to be enzymatically degraded (35). In addition, certain lipids, such as glycosidic ether lipids and hopanoids, are known to withstand degradation better than others (36). Hopanoids are cytoplasmic and membrane-associated pentacyclic triterpenoid lipids commonly found within certain aerobic and anaerobic bacterial groups; however, they are not found in eukaryotes or *Archaea* (37, 38). The hopane carbon backbone of these fatty acids is highly resistant to degradation (39). In fact, hopanoids are particularly notorious for their resilience to degradation and are considered among of the most common molecules detected in prehistoric samples worldwide (40, 41). Although their current function is unknown, ancient hopanoid variants are believed to be comparable to sterols in eukaryotes, as they seemingly enhance membrane stability and protect bacteria from environmental stresses such as changes in acidity and temperature (42, 43).

Additional protection against degradation has been found within the DNA itself, as genomes with high GC content, such as that from actinobacteria, for example, seem to be more stable and harder to degrade than their counterparts. This is usually attributed to the additional energy needed to break the pair's third hydrogen bond. Also, in the case of dormant microorganisms, DNA repair enzymes could be activated by spontaneous taphonomic mutations such as those induced by UV radiation (e.g., DNA cross-links and oxidation events) and may play a role in the longer preservation of their genomic integrity (26, 44, 45).

SOURCES OF ANCIENT MICROBIAL DNA

Molecular studies of ancient specimens have shown that under specific environmental conditions, intracellular DNA is preserved for long periods. Rapid freezing and rapid dehydration are among the most common taphonomic conditions (i.e., conditions that induce fossilization) that preserve organic matter. To date, ancient microbial DNA has been isolated from various sample types, including permafrost, halites, amber, bones, internal organs, dental pulp, and coprolites (Fig. 1), as is discussed in greater detail in this section.

Extreme Environments

Fossil ice and deep strata of permafrost represent environments that promote the preservation of ancient microbes and aDNA (46). The constant low thermal energy and water availability make metabolic activity, such as enzymatic degradation, a difficult task in these ecosystems. As a response to these conditions, microorganisms may be induced into a state of dormancy as a means for their long-term survival (44, 47–49). One of the most recent examples of microbial resilience involved the resurrection of a 30,000-year-old *Pithovirus sibericum* sample preserved in Siberian permafrost, the oldest eukaryote-infecting virus isolated to date, which also retained its infectivity in spite of such an extended dormant state (50). Microbial population studies of ancient permafrost are also important for determining the mechanisms by which microbial communities survive thousands of years in these ecosystems (26, 51–55). Furthermore, ancient variants of cold acclimation and cold shock proteins, as well as other key components of microbial activities, would provide valuable information regarding the evolution of metabolic processes and survival mechanisms for temperatures below 0°C (47, 52, 53, 56).

Other extreme ecosystems, such as those found in high-salt environments, are common reservoirs of ancient microorganisms that become entrapped in halite or “rock salts,” specifically in brine droplets (57). In addition, deep-sea sediments provide cold, anoxic, and UV-absent environments that may promote the preservation of microorganisms, including viruses. As with permafrost, isolation of ancient microbes in these ecosystems may provide evidence of survival mechanisms that could be environment-specific and highly conserved.

Amber

Amber possesses several properties that enable the preservation of ancient microorganisms (Fig. 1A and B), such as its low water activity and the presence of sugars in bacterial cell walls that may prevent cell damage during the dehydration process. The absence of water in amber makes it the ideal environment for enzyme inhibition and, thus, cell component

preservation, including DNA (58). Many bacterial isolates from amber belong to the genus *Bacillus*, which consists of aerobic spore-formers with a genome protected from UV radiation, oxidizing agents, and depurination by several mechanisms. Spores are protected by several different layers within the bacterial cell, are located where water percentages are the lowest, and exhibit the lowest pH values within the cell. Notably, even when these factors are modified, spore DNA remains conserved. This characteristic of spores has been attributed to the action of small, acid-soluble proteins (SASPs), encoded by *ssp* (59). SASPs are synthesized during the late stages of spore formation and are consequently degraded when spores germinate. SASPs are known to protect DNA by binding to and saturating the molecule. Five to ten percent of the total core proteins in *Bacillus* spores are SASPs, and spores lacking over 85% of these proteins are incapable of being protected from DNA-damaging agents, such as UV radiation (60). These compounds protect the DNA for extended periods.

Though it was previously assumed that the survival of ancient microbes was restricted to spore-forming bacteria, Gram-positive staphylococci and *Micrococcus* spp. have also been isolated from 23- to 35-million-year-old and 120-million-year-old amber, respectively (61–63). Even more unexpected was the isolation of Gram-negative bacteria resembling *Brevundimonas* in 25- to 40-million-year-old amber (64). The isolation of a Gram-negative, non-spore-forming bacterium suggests that microbes have an array of currently unknown mechanisms that could be involved in their long-term survival.

In the case of amber, the availability of nutrients during the initial stages of amber formation allow entrapped bacteria to utilize compounds such as succinic acid until they later enter a state of dormancy. Thus, such compounds represent potentially invaluable resources to maintain bacterial growth during short periods of adjustment. Currently, the specific molecular mechanisms by which microbes isolated from amber become dormant and are resuscitated after millions of years are being further investigated. Amplification of genes such as *rpf*, which stands for resuscitation promoting factor, and *luxS*, a gene involved in quorum sensing, may suggest that genes identified in extant bacteria may be involved in the survival of ancient microorganisms, including those preserved in amber (63, 64).

Bones and dental pulp

The recovery of bacteria from ancient bones and dental pulp is usually associated with disease (Fig. 1C and D). In terms of bone samples, this is probably because certain microorganisms can induce visible lesions during a chronic infection, and the resulting pathology can be used to identify potentially infected anthropological remains. Often, the location of the afflicted bone and the lesion type can be indicative of a specific disease; however, because diagnostic morphologies often look alike even to the trained eye, molecular verification of the diagnosis is necessary. Samples of ancient teeth are also important because large concentrations of bacterial and opportunistic pathogens tend to accumulate in the dental pulp of infected hosts and may therefore be preserved in the skeletal remains. For example, studies have detected *Yersinia pestis* and *Salmonella enterica* serovar Typhi in ancient teeth (65–67).

Internal organs

When adequately preserved, corporal tissue may provide vast quantities of information on the specimen. For example, DNA was isolated from the internal organ tissues of an Andean human mummy dating 1,000 to 2,000 years before present (YBP) (68) (Fig. 1E). The persistence of the original human and bacterial templates was assessed using an amino acid racemization test, quantification of mitochondrial DNA copy number, survey of 16S rRNA gene in the different organs, and sequence analysis of bacterial PCR amplicons of different lengths. Results showed that both the original human DNA and the DNA of the bacteria of the mummy gut were preserved. Sequence analysis of the 16S rRNA gene amplicons from the mummy colon showed that such sequences allowed for the detection of several species of the genus *Clostridium* that are part of the microbiota of the human colon (69). This work represented the first example of a methodological approach that is applicable, in principle, to any mummified human remains. This work also contributed to opening the way for studies on the structure of the human microbiota, ranging from prehistory to the present.

Analyses of 16S ribosomal DNA (rDNA) isolated from the internal organs of mummies have allowed scientists to determine the microbial diversity of these ancient digestive systems, as well as the degree of DNA preservation in these mummified samples. Cano et al. detailed anatomical, histological, and molecular investigations on the organs of a mummy from the Neolithic Age commonly known as “the Iceman” (5,350 to 5,100 YBP) (70). In this study, 16S rRNA gene fragments were amplified, cloned, and sequenced from various regions of the gastrointestinal tract, and the sequences were compared to those of extant bacteria. Results demonstrated that the detectable biota in the stomach was entirely composed of *Burkholderia pickettii*, an organism commonly found in aquatic habitats, most likely due to swallowing large amounts of water. The colon, on the other hand, contained several members of the fecal microbiota associated to humans, such as *Clostridium perfringens*, *Clostridium ghonii*, *Clostridium sordellii*, *Eubacterium tenue*, and *Bacteroides* spp. The Iceman’s colon, however, was also found to contain, rather unexpectedly, some members of the genus *Vibrio*. The authors used these data to reconstruct, in part, the last days of the Neolithic man’s life.

Coprolites

Coprolites are mummified or petrified feces and are perhaps one of the richest sources of ancient microbes (Fig. 1F). Coprolites have been found in dry, cold, and even tropical environments (71, 72). Early studies used morphological observations to determine the parasitological composition of coprolites. Similarly, the possible diet of the specimen was determined based on grains and seeds preserved in the feces. These morphological studies showed that helminth eggs, specifically those from *Diphyllobothrium* spp. and *Enterobius* spp., were preserved for thousands of years, although it was not clear if they were still infective. Paleoparasitological studies of coprolites were among the first to suggest that dietary habits, and even lifestyles, of ancient cultures (e.g., hunter-gatherer people vs. agriculturalists) could be discerned from ancient samples (73–76).

Selective molecular techniques applied to studies with coprolites have focused on both pathogenic and nonpathogenic microorganisms of interest (52). For example, attempts have

been made to characterize the bacterial profiles of fecal material inside fossilized human bodies. Bacterial DNA belonging to the *Alpha*-, *Beta*-, and *Gammaproteobacteria*, *Bacteroides*, and *Clostridia* groups was recoverable in such cases by PCR amplification. Other bacterial species, such as those belonging to *Eubacterium* spp. and *Vibrio* spp., were also detected (74). In addition, given that the gut microbiomes of modern human societies seem to be a reflection of diet and culture (77), recent paleomicrobiology studies have discerned dietary habits and even cultural differences by characterizing the coprolite microbiome using high-throughput sequencing of the 16S rDNA fragments (72). While coprolites possess a core microbiome that is comparable to extant human gut microbiomes, the proportions of certain microbial groups (both bacterial and fungal) have been found to be diet specific and thus may be ethnic group specific (76, 78).

Metabolites remaining in coprolites are also proving to be an important approach to detail diet. For instance, the metabolic degradation of plant products has recently been utilized to expand the concept of the Neanderthal diet. It was generally thought that Neanderthals had mostly a carnivorous diet, yet chemical analyses of coprolites are revealing that the Neanderthal diet included plant material as well (79). These results demonstrate not only that the presence or absence of DNA from specific microorganisms in coprolites may be important when deciphering diet but also that the metabolic pathways for the degradation of key compounds may be a tremendous source of information as well.

TECHNIQUES IN PALEOMICROBIOLOGY

There exists a wide variety of methods that have been or are increasingly applied in paleomicrobiology studies. These methods include culture-based approaches, microscopy, determination of fatty acid composition, PCR, sequencing, and high-throughput sequencing.

Culture Methods and Microscopy

Culture methods provide the advantage of isolating ancient microbes and distinguishing their metabolic capabilities. However, culturing microbes from ancient sources is a difficult task, since most microorganisms may not resuscitate in common laboratory media. Furthermore, experimental design should accommodate the growth of both expected and unexpected microorganisms based on sample type (46, 59). However difficult and seemingly unlikely, increasing numbers of attempts have successfully resuscitated microorganisms from prehistoric amber and permafrost samples (50, 64). Similar studies have pinpointed possible key genes in the resuscitation process, including those for various proteins that act as resuscitation-promoting factors (62, 80, 81). However, the overall gene expression cascade by which latent microorganisms respond to changes in the environment and resuscitate remains largely unknown.

Microscopy also provides the advantage of characterizing the morphology of ancient microorganisms, but often without the need of cultivation previous to observation. In addition, the technique has mainly been applied to the study of the parasitological composition of coprolites. In the case of doubtful objects found in coprolites, transmission electron microscopy is also helpful (82). Notably, morphological and biomolecular similarities may cause erroneous identification; thus, verifying morphological results with

molecular analyses, such as PCR, is recommended for a more precise characterization, as is the case of the parasite *Paragonimus westermani*, whose egg morphologies are commonly confused with those from *Diphyllbothrium latum* (83).

Fatty Acid Composition

Fatty acid profiling involves the characterization of the fatty acids comprising the cell membrane using chromatographic techniques, including liquid-, gas-, and solid-phase extraction and supercritical fluid extraction. A detailed description of these techniques can be found in other literature sources (84). Fatty acid composition studies rely on the ubiquity of phospholipids in the membranes of living cells. Fatty acids in the membrane of dead cells rapidly degrade; thus, the method also provides an estimation of the viable biomass in a sample (85). Among the most commonly utilized fatty acids for microbial profiling are fatty acid methyl esters (FAME), a type of fatty acid derived by transesterification of fats with methanol. Information from FAME studies enables the characterization of each individual fatty acid by the number of carbons and double bonds, as well as their type and location within the molecule. For example, bacteria from the genera *Staphylococcus*, *Listeria*, *Bacillus*, *Yersinia*, *Salmonella*, and *Escherichia* exhibit unique FAME profiles. Such studies have suggested that FAME characterization may be a unique fingerprint for differing bacteria. Moreover, it has been used to differentiate members of the genus *Bacillus*, including *Bacillus anthracis* and *Bacillus cereus* (86).

Fatty acid composition analyses have been shown to be useful when comparing ancient and extant isolates that show high 16S rRNA gene sequence similarity. The technique has been used to profile bacteria isolated from ancient amber and ice crystals and has successfully differentiated ancient and extant bacteria with similar 16S rRNA profiles, including *Bacillus thuringiensis* and *Bacillus sphaericus* (58). While it is not useful for the estimation of a bacterial isolate age, it does represent a way to confirm differences between strains.

DNA Extraction and Amplification

DNA analyses of ancient microbes may represent one of the most robust and powerful tools for paleomicrobiology. Perhaps the most critical step for studying ancient DNA is a successful extraction method. While the classical phenol-chloroform extraction was preferred and is still used (87), commercial kits present a quick, safe, and effective way of obtaining ancient DNA while reducing the possibility of both external contamination and cross-contamination. The type of DNA extraction kit to use will largely depend on the sample type, but different protocols and modifications to manufacturer kit instructions have been developed in order to optimize the quantity of DNA obtained from ancient samples (88, 89).

For nearly 25 years, the preferred method to analyze ancient DNA has been amplification by PCR (34, 90, 91). However, since aDNA becomes fragmented with time, it could be difficult to amplify large regions of the gene of interest. Thus, it is preferable to design primers targeting small overlapping regions of the gene, preferably below 200 bp in length (92). The most common microbial genes targeted include highly conserved genes such as the 16S rRNA gene and *rpoB* (65). Primer design should also target various key virulence genes for

more robust and precise results. This is especially true when detecting *Mycobacterium tuberculosis* complex and *Mycobacterium leprae* in ancient bones, since certain environmental *Mycobacterium* spp. are present in soil (93–95). As with any archaeological sample, soil and its associated microbiota will most likely be present as contaminants; thus, it is crucial to ensure optimal sample handling and, whenever possible, discard the exterior layers of the sample, focusing all analyses on sample cores, which contain less of the environment-associated microbiota (76).

Notably, many fossil samples possess PCR inhibitors that may induce false-negative results, such as tannins, porphyrins, and hematin. In addition, taphonomically induced chemical modifications, such as DNA cross-links, may also inhibit or interfere with amplification reactions by PCR and should be taken into consideration when analyzing aDNA. For example, adding *N*-phenacylthiazolium bromide helps break cross-links between DNA strands (25). PCR inhibition is easily determined by adding an aliquot of the sample aDNA to a positive control in the amplification reaction. Detected inhibition can be diminished by using the diluted PCR product as a template in serial amplifications as well as by using bovine serum albumin in the reaction solution (96, 97).

Other useful techniques following the same principle of gene amplification include terminal restriction fragment length polymorphism and quantitative PCR. While terminal restriction fragment length polymorphism has mainly been applied to characterize microbial communities in diverse environments (98), it has also helped understand the microbial composition of ancient samples (57, 72). In contrast, quantitative PCR studies have been useful in determining the quantities of ancient microbial DNA obtained from paleosamples, especially in nonpermafrost sediments (98).

Sequencing-Based Techniques

The amplification of a gene of interest in aDNA studies should be followed by further sequencing analyses in order to confirm results and obtain comparable data. The detailed description of aDNA amplification and sequencing can be found in other literature sources (99). Recent technological advances during the last 10 to 15 years have substantially improved sequencing techniques and expanded public databases (100). In addition, sequencing of PCR products of ancient microbes enables the estimation of evolutionary changes of the targeted gene.

High-Throughput Sequencing

The microbial communities present in a variety of extant ecosystems (e.g., soil, rotting wood, fecal matter, and the human microbiome) are increasingly being characterized using high-throughput sequencing. aDNA studies have also begun to apply metagenomic techniques to increase the coverage of their molecular analyses, revealing information that could not be assessed by culture methods, microscopy, or sequencing of a specific gene alone (78, 101, 102). For example, the microbial profiles of coprolites from two extinct Caribbean cultures have been successfully characterized by next-generation sequencing of 16S and 18S rRNA genes, revealing information on the state of fecal microbiomes prior to the arrival of Europeans to the region (76). Similarly, next-generation shotgun sequencing

(i.e., the untargeted sequencing of the total DNA fragments present in a sample) is currently being applied to understand not only the composition of microbial communities in ancient samples but also the metabolic pathways represented by key functional groups (16). The application of shotgun sequencing has already revealed the composition and complexity of bacteria, eukaryotic and prokaryotic viruses, fungi, and archaea in ancient samples. Such data could reveal important microbe-microbe and microbe-host interactions, as well as key metabolic processes preserved in these ancient microbiological communities.

WHAT ARE ANCIENT MICROBES TELLING US SO FAR?

Insights into Ancient Diseases

The isolation of pathogenic microorganisms from animal and human samples can provide information about the pathogenicity, origin, phylogeography, and evolution of the targeted microorganism, a process also known as retrospective disease diagnosis (35, 103). The epidemiology of ancient infectious diseases, especially in human populations, could provide important information regarding nutritional status, as well as the population density and hygienic conditions for spreading. Some of the best-studied ancient pathogens include *M. tuberculosis* and *M. leprae*, the causative agents of tuberculosis and leprosy, diseases that have afflicted humankind for thousands of years. DNA belonging to these pathogens has been detected in ancient skeletal remains found in various geographical regions of the world; however, their history of infection is still debated (104–110). As previously discussed, mycobacterial DNA seems to be particularly resistant to degradation due to its high guanine-cytosine content as well as the high mycolic acid content in their cell walls (35, 111, 112).

Mycobacterium tuberculosis infections were widespread in the past and represent a reemerging global threat since the beginning of the decade; therefore, it is important to understand this organism's origin and genome evolution (113–121). DNA and biomarker studies have demonstrated that tuberculosis (TB) has existed for over 4,000 years, as it has been detected in samples from ancient Egypt, ancient Rome, and pre-Columbian America (35, 122). Early studies indicated that *M. tuberculosis* may have originated in Europe and perhaps the Far East as a cattle-associated pathogen that later switched to human hosts. *M. tuberculosis* complex DNA was detected in bones of a longhorn bison dating over 17,000 YBP preserved in a cave in North America (123), fueling the hypothesis that infected bovines could have been the source of the disease in America during their migrations across the Bering Strait (35). However, phylogenetic studies comparing the bison TB strain to a 9,000-year-old human TB strain concluded that *M. tuberculosis* might have preceded *Mycobacterium bovis*.

The history of TB has proven to be far more complex than previously expected, however. For example, ancient *M. tuberculosis* strains detected in Egyptian mummies showed greater similarities to *Mycobacterium africanum* than to *M. bovis* (124). Also, although *M. bovis* was previously considered mainly a bovine-selective pathogen, putative cross-infections have been detected in human samples corresponding to the Iron Age in Siberia (125). In addition, and further adding to the complexity of the story, a recent study suggests that migratory seals could have also played a key role in distributing African *M. tuberculosis*

infections to the pre-Columbian New World (126). Consequently, further studies on ancient *Mycobacterium* spp. are necessary to clarify the phylogeographical history of TB.

Another disease that has ailed humankind for centuries is leprosy, otherwise known as Hansen's disease, caused by *M. leprae*. In fact, despite the success of current multidrug therapy, World Health Organization statistics for 2013 reported a global registered prevalence of this disease at 189,018 cases (127). The pathogen was apparently widespread across Europe between the 14th and 16th centuries, as deduced by its increased detection in skeletal remains (128, 129). One particular study carried out in three separate laboratories was able to detect *M. leprae* aDNA in 1st-century tuberculosis-positive skeletal remains from the Tomb of the Shroud in Jerusalem (130). However, the oldest leprosy bacillus DNA detected to date is most likely from skeletal remains in India dating 4,000 YBP (131). Phylogenetic studies suggest that American *M. leprae* strains originated from Europe; however, the epidemiology, geographical origin, and distribution of the leprosy bacillus throughout the Old World are still being elucidated (107, 108, 129). Research also suggests that, similar to other *Mycobacterium* species, *M. leprae* was probably initially free-living and underwent a bottleneck event suspected to have reduced its genome to its current state of obligate intracellular pathogenesis within humans and wild armadillos (132).

Though not as well studied as *M. tuberculosis* and *M. leprae*, another example of an ancient human pathogen that leaves visible skeletal lesions, or "*caries sicca*," is *Treponema pallidum*, the causative agent of treponemal diseases such as venereal syphilis, yaws, pinta, and endemic syphilis (bejel). The history and origin of the modern syphilis strain in particular have been the source of controversial debate for years and still remain one of the greatest mysteries in paleomicrobiology and modern medicine. Theories of its origins include the Columbian hypothesis, stating that treponematoses originated in the Americas and was brought to Europe with Columbus's return, and the pre-Columbian hypothesis, stating that different strains of *T. pallidum* evolved alongside various civilizations across the globe, but the disease was often misdiagnosed.

Initially, hardly any skeletal remains retrieved from pre-Colombian Europe showed *caries sicca*, whereas putative syphilis-positive samples from the Americas were abundant, including treponematoses-like lesions in pre-Columbian Antillean indigenous cultures (133–135). This fueled the belief that syphilis originated in the New World and was taken to Europe after contact with the Americas. However, osteoarchaeological studies have detected treponematoses-like lesions in pre-Columbian crania from Israel and Europe, as well as in teeth from 18th-century Austria, thus supporting the pre-Columbian hypothesis. This suggests that syphilis may have been present in the Eastern Hemisphere long before Christopher Columbus departed for the New World, but had gone largely unnoticed, probably due to a less virulent state or misdiagnosis of the disease (136–138). However, lesions caused by yaws, endemic syphilis, and venereal syphilis have similar morphologies and could even be confused by a trained eye; therefore, osteoarchaeological studies should include molecular analyses such as microbial paleogenetics and immunological assays (123, 139–142). Interestingly, some even consider the detection of aDNA from the syphilis microbe improbable due to the epidemiology of this pathogen after years of infection; however, further studies are necessary to determine if this is correct (143–145).

Other ancient diseases, such as septicemia caused by *Escherichia coli* and *Y. pestis*, as well as protozoan and viral infections, are revealing that these pathogens and the conditions they induce are not recent events (65, 94, 146–149). For example, data attribute numerous ancient epidemics to *Y. pestis*, which we now know were in fact caused by different strains that spread across the Mediterranean, Europe, and Asia at different time points (150, 151). *Y. pestis* has also been confirmed as the causative agent for the Justinian Pandemic in the 6th to 8th centuries, presumably travelling from Asia over the Alps and through Europe (152, 153). Furthermore, reconstruction of the genome of the European Black Death agent suggested that modern epidemic *Y. pestis* strains may have derived from this 14th-century variant (154). These observations demonstrate how phylogenetic analyses of aDNA shed light on possible evolutionary processes (mutations, speciation events, origins, and acquisition of ancient antibiotic resistance) and help elucidate disease migration patterns.

Studying the dispersal and host-microorganism co-evolution of these ancient pathogens is important to understand the mechanisms by which these pathogens interact with humans and animals; it also helps in the search for new medical treatments. For example, information gathered from both environmental and animal aDNA studies could provide insight into genome evolution and composition prior to the widespread use of antibiotics, as well as the acquisition of virulence factors and other mobile elements that are present in their modern-day relatives (155). Very few studies have attempted to identify such genes in ancient samples. For instance, antibiotic resistance has long been associated with the relatively recent advent of antibiotic use. However, aDNA studies have shown that antibiotic resistance is not modern and is not restricted to clinical settings. Further studies are needed to determine the mechanisms by which antibiotic resistance genes, such as *vanA*, act in the environment and if natural sources of vancomycin-like molecules are promoting bacteria to acquire genes encoding such resistance (156). Data regarding this matter is of unquestionable importance for clinical microbiology and the future of antibiotic development.

Ancient Microbial Life and Evolution

Although paleomicrobiology has mainly focused on ancient human pathogens, aDNA studies have begun to focus on saprophytic microbes and entire microbial communities preserved within these samples. For example, aDNA and morphological studies of the 30,000-year-old *Pithovirus* pinpointed a hybrid array of characteristics from modern-day Pandoraviruses and icosahedral DNA viruses (157, 158). However, how and when these genetic events took place remain unknown. Paleomicrobiology could also help determine how present-day bacterial strains have acquired eukaryotic characteristics, such as double-membrane cellular compartmentalization, the capacity of endocytosis (observed in *Gemmata obscuriglobus*), and the presence of microtubules (159–162).

Recent large-scale analyses of the modern human microbiome have also proven useful in aDNA studies. For instance, the composition of the modern human microbiome has revealed that inhabitants of the gut may be influenced by culture and thus differ according to geographical region (77). Diet, unquestionably influenced by culture, also has a tremendous impact on the composition of the gut microbiome. These findings provide the basis for

understanding how paleo diets influence the microbial composition of coprolites, thus revealing cultural differences, even from groups that coinhabited a particular region (72). While differentiating ancient cultures has mainly relied on archaeological artifacts, the microbial composition of coprolites has supported archaeological data, therefore opening the opportunity of using paleomicrobiology to study the heritage of ancient cultures. In fact, the future of paleomicrobiological studies will probably include a more interdisciplinary approach, combining archaeology, anthropology, paleogeography, paleobotany, and paleozoology metadata to obtain a better understanding of a specimen's history.

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In modern specimens, changes in the soft tissue of the corpse, insect succession, and alterations in the composition of nearby soil (e.g., pH and oxygen levels) are “rate of exchange” methods often used to assess the postmortem interval (PMI) in an investigation (163). In addition, the potential use of corpse and soil-associated bacteria as indicators of PMI is being studied (164). Similarly, paleoforensics could also rely on key microbial structures as PMI indicators. Among the best-studied possibilities are hopanoids, also known as “orphan biomarkers,” which have been proposed as molecular fossils due to their high preservation and ubiquity in microbial communities (165, 166). (For more details on hopanoids, see “How Can DNA Be Detected after So Long?” above) Although further studies are necessary in order to fully understand the distribution and production of hopanoids within different bacterial genera, these molecules could be useful in paleoforensics since their structural variations may indicate the presence of certain bacterial groups within the sample. In fact, changes in the concentrations of these biomarkers could help us infer the PMI of a specimen and how its associated microbial community profiles may have changed through time.

Given the apparent higher resistance of certain microorganisms towards DNA degradation, DNA fragments of such microbes could serve as potentially useful “molecular fossils” in paleoforensic studies. DNA degradation behavior is currently being studied, and *in vitro* experiments have already determined mycobacterial DNA to be far more resistant to heat degradation than eukaryote DNA (167). Therefore, understanding DNA degradation patterns in bacteria could help us determine potential key genes or genetic fragments as indicators of PMI as a function of their degradation through time.

CONCLUDING REMARKS

Paleomicrobiology is revealing important aspects of microbial resistance and evolution. The field has been mainly associated with the study of human and plant pathogens, opening the opportunity to investigate the evolution of ancient diseases and how these affect us today, yet the field is not restricted to pathogenic microorganisms. Normal microbiota of different ancient ecosystems, including permafrost, halites, bones, dental pulp, amber, fecal masses, and coprolites, aid in our understanding of microbe-environment and microbe-host interactions. Methods such as microscopy, culture, PCR, and sequencing have only provided a peripheral view of ancient microbes, but the application of high-throughput sequencing

techniques has shown that molecular paleomicrobiology has the potential to provide more information than previously expected.

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FIGURE 1.
Sources of ancient DNA. (A) Bee entrapped in amber; (B) plant material entrapped in
amber; (C) bone; (D) teeth; (E) tissue; (F) coprolites.