PALAEOMICROBIOLOGY OF HUMAN INFECTIOUS DISEASES

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Summary

Palaeomicrobiology is the study of ancient infectious diseases, based on the examination of archaeological or historical specimens. This chapter deals with human infectious diseases, but the field also covers diseases of other animals and plants. Its recent development dates from the introduction of molecular methods, mainly the amplification and detection of ancient DNA from disease-causing microbes, known generally as pathogens, but also includes studies of microbial carbohydrates, lipids and proteins.

Why study palaeomicrobiology? Originally the aim was to verify diagnoses reached by palaeopathologists after the careful study of changes to bones and mummified tissues. It was soon realized that palaeomicrobiology provides answers to historical questions, such as whether European colonialists brought tuberculosis to the Americas. Concurrent developments in genomics have enabled comparison of gene sequences from ancient and modern pathogens. This has led to increased understanding of the origin of infectious diseases and their spread around the world. This is important today, as microbial pathogens are continuing to be a major problem to humanity, with increased levels of infection, ability to cause illness and death (virulence) and drug resistance.

1. Introduction to Infectious Diseases

Microorganisms are found everywhere on Earth. The vast majority live in the environment, but some have adapted to living on or in higher organisms such as animals and plants. The human commensal microflora comprises the microorganisms that inhabit every part of our body with access to the external environment. It consists of a complex community of microorganisms, predominantly bacteria. Different sites, such as the mouth, skin, gastro-intestinal and genital tracts, have their own distinct microflora, which is believed to be important in providing micronutrients, preventing colonization by disease-causing pathogenic microbes, and sometimes causing disease in a susceptible human host. It is believed that members of the human commensal microflora have co-evolved with us.

However, some species of microbes have a different lifestyle and are described as pathogenic. They infect the host, cause damage and sometimes death, and maintain their population by transmission from one human host to another. This may be direct, via other animal hosts, or via vectors such as insects or mollusks, for example. In order to understand infectious diseases we need to know about the different types of pathogenic microbes and the various strategies they have adopted to survive in their human host.

1.1. Principal Groups of Microbial Pathogens

Human pathogenic microorganisms include bacteria, viruses, unicellular and multi-cellular parasites, and fungi. Bacteria are normally microscopic single cells with a simple structure described as prokaryotic. Most have a cell wall, all have a nuclear region that contains DNA, there are ribosomes that contain RNA and there may be

additional structures such as flagellae, fimbriae (hairs) and storage granules. There are two major groups of bacteria defined by their cell walls: Gram-positive bacteria have a thick wall predominantly of peptidoglycan; Gram-negative cell walls have a thinner layer of peptidoglycan, plus an outer membrane. These characteristics correlate with important taxonomic differences between these two groups and confer a different susceptibility to antibiotics. Some Gram-positive bacteria produce very resistant endospores that enable the organism to survive extreme environmental conditions. Examples are *Bacillus anthracis* that causes anthrax and *Clostridium botulinum*, which produces a toxin that causes botulism. Gram-negative bacteria include several groups of pathogens including the causative organisms of bubonic plague, brucellosis or undulant fever, typhus and typhoid fevers. A significant group of bacteria have a very lipid-rich and complex cell wall that is associated with resistance to environmental extremes and sometimes with very slow growth. These are the mycobacteria that include the important human pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae* – the causative organisms of tuberculosis and leprosy, respectively. The majority of bacterial pathogens can survive and grow in the environment but a few are obligate pathogens and require a living host in which to grow. Examples include the bacteria that cause leprosy and typhus fever.

Viruses are non-living entities that cannot replicate in the absence of a host cell that provides the necessary environment and mechanisms. They contain either DNA or RNA, which may be single or double-stranded. This is surrounded by a protein capsid. Virus particles may be naked or acquire an outer envelope from the host cell in which they replicate. Some viruses are able to integrate their genome into that of their host cell and may thereby become latent. Viral diseases include the common cold, influenza, measles, hepatitis and smallpox.

Parasites and fungi are higher order organisms (eukaryotes), with a cellular structure that is analogous to that found in animals and plants. Parasitic infections caused by unicellular organisms (protozoa) include malaria, Chaga's disease, and leishmaniasis. Multicellular parasites include insects (fleas, lice, ticks, etc) that are often important in spreading other diseases. Other multicellular parasites include flukes – the cause of schistosomiasis (bilharzia), round worms (e.g. *Trichuris* sp.) and flat worms (e.g. the tapeworm *Taenia* sp.) – common in intestinal infections. Fungi are major pathogens of plants but they occasionally cause human infections. Organisms such as *Candida albicans* are part of the human commensal microflora. *Candida* spp. normally have yeast morphology but they can become invasive, especially in a host with increased susceptibility to infection. Occasionally fungi can cause systemic infections that can be fatal, such as coccidiodomycosis and histoplasmosis. Antibiotics designed to act against bacteria are useless in the treatment of viral, fungal or parasite infections.

1.2. Different Relationships of Microbial Pathogens with Humans

It is helpful to consider the parasitic lifestyle from the viewpoint of the microorganism, which needs to survive within its host, multiply and be transmitted to another host. Commensal bacteria cause minimal disruption to their host and are acquired shortly after birth by passage through the birth canal and subsequent close contact with the mother. However, pathogens can only be maintained in a population if they cause a

chronic or latent infection, or if they can readily be transmitted to another susceptible host. In this case the population must be sufficiently large for the pathogen to be maintained. In recent times measles virus has died out in remote island communities, and poliovirus is near global elimination due to an active vaccination program that has reduced significantly the number of susceptible individuals.

Looking at human prehistory, we believe this was a society of gatherers and hunters, with no fixed settlements and small family groups. Similar nomadic societies today show evidence of long-lived intestinal parasites such as *Taenia* spp. These are infections acquired early in life and which can persist for years. Tuberculosis is another example of an infection which is believed has co-existed with the human species over a prolonged period of time. The pathogen *M. tuberculosis* is normally contained by the host cell- mediated immunity, illustrated by the surprising statistic that only approximately 10% of infected adults develop an active infection. However, active infection can develop in early life (< 5 years), extreme old age (Section 6.1) or when external circumstances cause immunosuppression (Section 6.2). This scenario of prolonged latent infection, with occasional opportunities for activation and transmission, enables the pathogen to persist even in small or low-density populations.

The change of human society to farming and settlements, known as the Neolithic transition, was associated with larger, denser populations, widespread animal domestication, a more sedentary lifestyle and greater social stratification. High population densities are associated with rapidly transmissible viral diseases such as measles, mumps, chickenpox and smallpox. Gastro-intestinal infections are another group of diseases that are found in densely populated human settlements with poor sanitation. Recently it has been discovered that more virulent strains of *M. tuberculosis* are found in cities with a long history of human habitation, suggesting that increased opportunities for transmission may be a factor. Agriculture subsistence, surprisingly, is linked with a fall in overall human nutrition due to a reduction in the variety of foodstuffs. Under such conditions there is increased susceptibility to opportunist infections from members of the commensal microflora or low level pathogens. Animal domestication and land clearance increases the opportunities for humans to acquire infections from animals. In such zoonotic infections humans are accidental hosts and are not essential for the perpetuity of the pathogen. This may be the reason why some zoonotic infections are highly virulent for humans, such as those caused by Ebola, Herpes B and Lassa viruses.

2. Detection of Molecular Markers of Microbial Pathogens

Detection of infectious diseases in human remains requires a different expertise from the examination of disease in living patients. The usual specimens available for study are bones. There may also be mummified tissue, teeth, nails and hair. Bones may show signs of inflammation, such as destruction of tissue caused by abscesses in an acute infection, and new bone formation associated with chronic inflammation. Imaging can indicate internal lesions, ranging from a gross to a microscopic scale. However, all these methods require independent confirmation, which can be provided by various categories of molecular markers.

2.1. Overview of Different Molecular Markers

An infectious disease is the result of an interaction between a host and a pathogen. Infection alone will not always cause disease, as shown by the high proportion of the global population who are infected with infectious agents, such as cytomegalovirus, Epstein-Barr virus and *M. tuberculosis*, but show no symptoms unless immuno-compromised. Disease results from damage to the host tissues when the immune response is unable to control an infection, either during an acute phase of active infection, or a chronic infection that persists over time with occasional active episodes. Damage can be caused directly by the pathogenic microbe, by the host response to the pathogen, or by a combination of both. Therefore, past diseases can be detected by seeking molecular markers of the pathogen itself, its metabolic products, or specific host responses to a particular pathogen.

Most non-viral pathogens have a basic structure of a cell wall, nuclear material and cytoplasm. There may be other structures such as bacterial endospores, parasite eggs, or fungal spores and hyphae. Over time these structures will degrade but there may be detectable molecular traces left behind in infected sites. Some pathogenic bacteria produce toxins that may be detected in tissue. Activation of the human humoral immune response results in production of circulating antibodies. Both toxins and antibodies are potential host biomarkers of infection.

Another important feature that can be detected is bone re-modeling following disease resolution. This is visually detectable in old skeletal remains and in some infections the bony changes are capable of providing a diagnosis. The chronic inflammation associated with pulmonary tuberculosis leads to the production of granulomas and calcification of tissue, which can still be detected after death of the host.

2.2. Persistence and Changes over Time

The study of the processes involved in the changes that occur after death in animal and human remains is known as taphonomy. It spans the timescale between forensic anthropology and palaeontology. It is necessary to understand the relative persistence of different molecular markers to post-mortem changes in order to choose appropriate molecular markers for the study of ancient infectious diseases. In addition to the differential persistence of the various categories of molecular markers, the site within the body is an important factor. Finally, the external environment of the human remains has a profound impact on the preservation of markers over time.

The Ancient Egyptians had a good understanding of post-mortem changes and realized that for successful embalming it was necessary to remove the internal organs. They left the heart in place, as this was believed to be the seat of intelligence and emotion. The natural decay process is initiated by the gut microflora, which is circulated around the body in an ‘agonal bacteremia’ due to loss of barrier function of gut capillaries at the time of death. Therefore sites remote from the gut and with limited vascularization have a slower rate of decay. Calcified sites are particularly resistant, especially the dental pulp area within teeth.

The environmental conditions influencing the rate of decay are the basic parameters that regulate the rate of chemical and enzymatic reactions. Therefore, reactions are faster at higher temperatures, up to a maximum at which they can occur. There will be an optimum level of oxygen, hydration and pH (acidity and alkalinity). If the external environment changes over time, it is likely that conditions for decay will occur more frequently than if the environment is stable. In general, stable, cold and dry conditions will facilitate long-term persistence. Molecular markers can persist in a wet environment but only if it is extremely cold or in the absence of oxygen and/or the presence of inhibitory substances. An example of such an environment is a peat bog. Recently Korean 'wet' mummies have been found with excellent preservation, apparently facilitated by lack of oxygen and the presence of lime. Molecular markers can be found in hot desert conditions, provided that the humidity is extremely low.

2.3. Choice of Specimens, Sampling Site and Molecular Markers

In palaeomicrobiology it is essential to remember that pathogenic microbes are not evenly distributed within the host during life and the same holds true after death. It is necessary to understand the nature of the infectious disease so that the likely sites for the detection of the causative organism can be identified and examined. For example, in a disease such as smallpox there are skin lesions, so dried skin may be a source of molecular components of the virus. Tuberculosis usually occurs in the lungs, so mummified lung tissue, calcified pleura (the membrane lining the chest cavity), or the inner surface of ribs are excellent sites to sample. Invasive pathogens that are disseminated around the body via the bloodstream can be detected in sequestered sites such as the heads of the long bones or the space formerly occupied by dental pulp. Markers of typhus fever, bubonic plague and relapsing fever have been detected from the dental pulp region and markers of malaria can be found in mummified muscular tissue and bone. Ancient feces known as coprolites are excellent sources of intestinal pathogen molecular markers and parasite eggs. Bones may exhibit areas of new bone formation (periostitis), or destructive lesions associated with abscess formation. Here the sites to sample are the leading edge of any lesion or re-modeled bone, and slightly beyond. Microbial biomarkers are often found on the outer surfaces of bones such as ribs, where there may be residual traces of infected tissue. This is an important difference from studies of human DNA, where the outer bone surface is often routinely decontaminated or removed, to avoid external contamination from other sources of human DNA.

In the modern day the classic pathology observed in museum specimens is seldom seen, due to the use of antimicrobial agents such as antibiotics. However, even before the development of effective treatments, it is important to realize that in most diseases, bony lesions were rare. In order to develop the pathogen must have spread around the body, and yet its human host must have survived for a sufficient length of time to allow the lesions to be formed. In the case of tuberculosis it is estimated that bony changes occurred in only 5–6% of all infections. Therefore, in the majority of cases of ancient or historical tuberculosis there were no lesions at all. Where written records are available, the historical context is important. There may be religious or civic archives, such as in the Ancient Egyptian and Babylonian civilizations. Some records exist that describe the location of mass burial sites due to plague, for example. There are Sanskrit and ancient

Chinese texts that describe a disease consistent with leprosy and we know that individuals thought to have this disease were often kept in social isolation (Figure 1). To study pathology in prehistoric times, indirect evidence of human remains with higher potential of infection is often sought. For example, artifacts in grave goods such as a crutch or stick may indicate a physical indisposition. Exclusion of graves from burial grounds has been interpreted as evidence of social stigma. Where re-burial of bones in an ossuary is the norm, failure to do so may indicate fear of infection. Using indicators such as this may be helpful in identifying individual remains or populations to be examined for particular infections.

The choice of molecular marker reflects a balance between availability, and the information that can thereby be generated. DNA is not especially resilient and can readily be broken down. However, DNA analysis enables determination of strains and lineages of infective microbes, which may elucidate pathogen evolution and the relationship with the human host. Carbohydrates and proteins are chemically more stable. Structural microbial components are not always specific but may enable analysis of the host response by antigen or antibody detection. Bacterial exotoxins, such as those that cause botulism or tetanus, are not stable in the external environment but antitoxins may possibly be detectable in human remains under suitable conditions. Mycobacterial cell wall lipid biomarkers are the most resistant to date, due to their very high molecular weight and extreme hydrophobicity.

Figure 1. Miniature of a leper with his bell from a Mediaeval manuscript, indicating the social isolation and stigma experienced by those suffering from this disease (Wellcome Library, London)

3. Traditional and Non-DNA Methods Used In Palaeomicrobiology

Before the development of methods based on nucleic acid amplification, ancient diseases were detected by a combination of visual appearance and traditional pathological techniques such as histopathology and immunology (Figure 2). Nucleic acid amplification led to an explosion of interest in palaeomicrobiology, and although work based on these earlier technologies has continued at a low-level, this area remains somewhat neglected and is ripe for further studies based on a new generation of methodologies such as those based on nanotechnology.

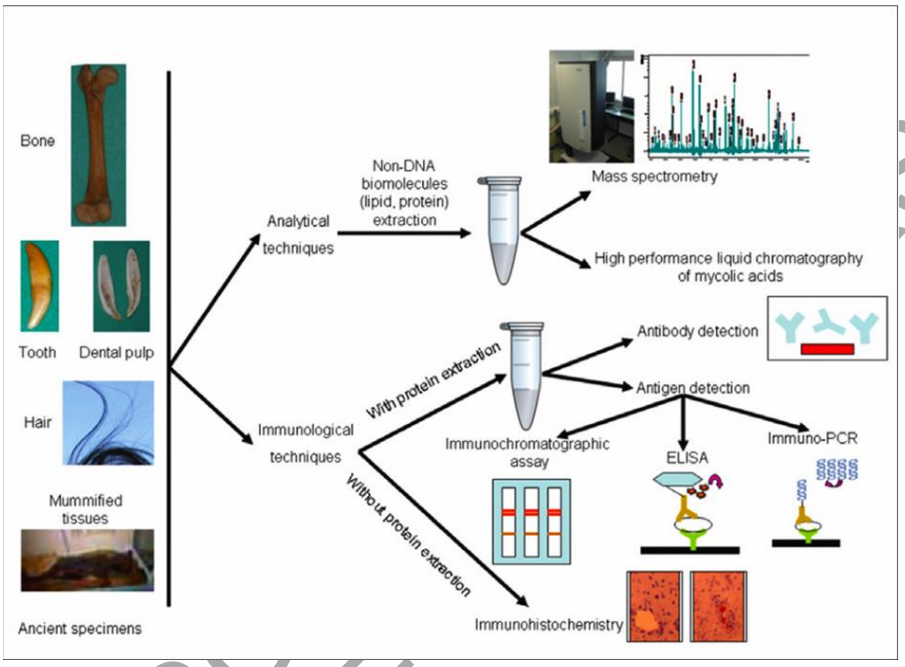

Figure 2. Source materials and methods used for nonnucleotide biomolecule detection in palaeomicrobiology (From Beyond ancient microbial DNA: nonnucleotidic biomolecules for paleomicrobiology. Thi-Nguyen-Ny Tran, Gérard Aboudharam, Didier Raoult, and Michel Drancourt, *BioTechniques* 50:370-380 (June 2011) doi 10.2144/000113689)

3.1. Palaeopathology, Imaging and Microscopy

Careful examination of bones can indicate the age of individuals and sex – normally post-adolescence. The development of palaeopathology has resulted in a consensus on how to evaluate skeletal material and apply differential diagnoses of the cause of observed lesions. The skeleton can exhibit lesions reflecting the experience of the individual during life, such as cancer, infection, malnutrition and trauma. Some infectious diseases result in such characteristic changes to the skeleton that it is possible to identify the disease. For example, the collapse of the spine seen in Pott's disease signifies tuberculosis. This was recognized in the early days of Egyptology so we know that tuberculosis was present in ancient Egypt. Other typical skeletal changes are seen in syphilis (Figure 3) and leprosy (Figure 4), but the differential diagnosis between these

two diseases requires a good knowledge of palaeopathology. Other skeletal changes can indicate an acute or a chronic infection, but not the specific cause.

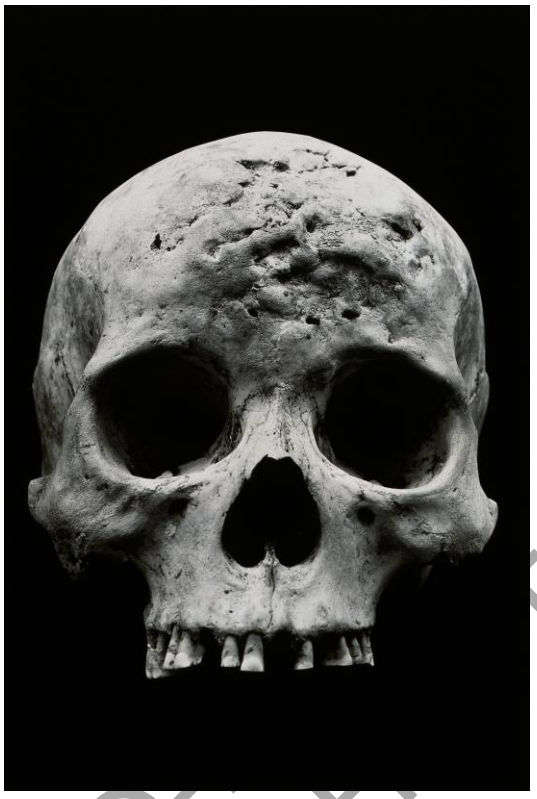

Figure 3. Skull of Alaskan Inuit, effects of syphilis, 1910 (Wellcome Library, London)

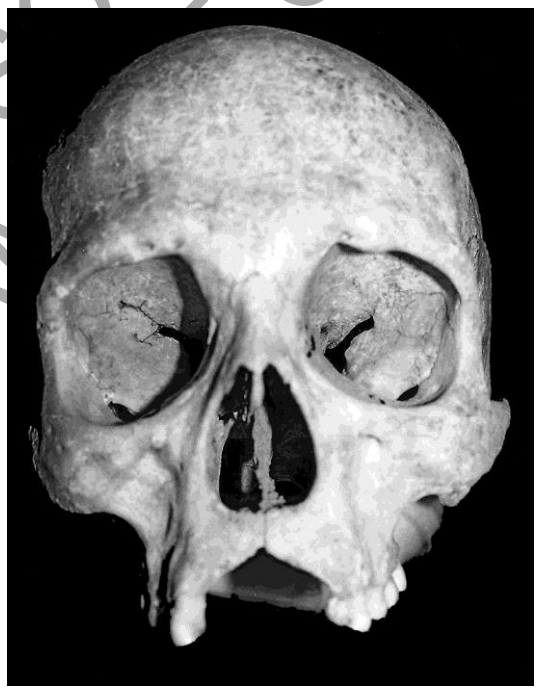

Figure 4. Skull from Byzantine Turkey, effects of leprosy (H. D. Donoghue)

Radiographic imaging has been used to augment visual examination of human remains since the late nineteenth century, when X-rays were discovered. One of the first applications of this new technology was the examination of ancient Egyptian mummified remains that had not been unwrapped. Imaging is useful in enabling lesions to be examined, including areas covered by overlying bone. Computerized tomography (CT) is a highly important development as it enables a three-dimensional image to be obtained. X-rays and CT scans have enabled sites of lesions and trauma to be visualized in mummies and bog bodies. When coupled with endoscopy, targeted biopsy is possible within preserved bodies. Bone densitometry, such as dual-energy X-ray absorptiometry (DXA) scans, can indicate changes associated with metabolic or infectious diseases.

Microscopy and histology of mineralized or preserved tissues can elucidate underlying pathology that is occasionally associated with likely pathogens. The first evidence of pre-Columbian tuberculosis was demonstrated in a child mummy from southern coastal Peru dating from around 1300 years ago. There was evidence of chronic disease in bone and soft tissue consistent with tuberculosis, and bacteria with similar morphology to *M. tuberculosis* were extracted from the tissue and visualized using the light microscope. Staining of *M. tuberculosis* is only possible if the microscope slide is heated to make the bacterial cell wall more permeable. In the Ziehl-Neelsen staining reaction, after heating and staining the smear with a red dye, it is decolorized using sulfuric acid with alcohol and counterstained with a green or blue dye. This early study demonstrated that the bacterial cell wall remained intact and acid-fast.

Preserved skin may show visible lesions caused by infections. A pre-Columbian mummy of a young man, also in southern Peru, was found to have numerous granulomatous lesions and vesicles on his skin. Giemsa staining and light microscopy plus electron microscopy showed typical clumps of bacilli with morphology similar to *Bartonella bacilliformis*. This causes Carrións disease, which is spread by sand flies and can cause acute fatal anemia or a condition known as verruga – characterized by wart-like lesions. Skin lesions may also indicate a viral infection. For example, there are vesicles on the face of the 20th Dynasty Egyptian Pharaoh Rameses V that may be due to smallpox. In mid 16th century Italy a Neapolitan mummy of a two year old showed typical pustules on the face. Samples of skin were rehydrated and examined using electron microscopy by negative staining and thin section. Structures with the typical appearance of smallpox virus were observed and confirmed by immunofluorescence (Section 3.2).

Visual analysis and microscopy can detect direct evidence of infection such as the presence of eggs produced by parasitic worms. For example, the eggs of the fluke *Schistosoma haematobium* were identified in an ancient Egyptian mummy in 1910. Over 80 years later, microscopy was used to determine a 65% prevalence of *S. haematobium* in a population on the border of modern Egypt and Sudan, dated between the 4th and 6th centuries. Electron microscopy has shown larval forms of the threadworm *Strongyloides* in the eviscerated intestines of the mummy of Asru, a priestess of Amun at Thebes who lived *circa* 1300 years ago. In addition, she had schistosomiasis, and there was a hydatid cyst of the tapeworm *Echinococcus granulosus* in her lung. Electron microscopy has demonstrated filarial worms within the muscle tissue of the Egyptian mummy Natsef Amun, who lived around 3100 years ago. Eggs of

the roundworm *Ascaris lumbricoides* and the fish tapeworm *Diphyllobothrium latum* were identified in a 13th century cesspool from the crusader city of Acre, then part of the Frankish kingdom of Jerusalem. Before this time the fish tapeworm was common in northern Europe but was not found in the Mediterranean Near East. It appears that pilgrims and crusaders who were infected with this tapeworm were responsible for bringing the parasite to this geographical region.

It is more challenging to detect single-celled (protozoal) parasites as they are very unlikely to persist and are microscopic in size. However, insects can occasionally be well preserved in amber, thus allowing even intracellular structures to be seen. Sand flies are vectors for the group of parasites known as trypanosomids, and a new genus has been identified from the proboscis and alimentary tract of a blood-filled sand fly found in Cretaceous Burmese amber. The amber beds have been dated to the Early Cretaceous –100–110 million years ago.

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Biographical Sketch

Helen Donoghue received her Special Honours B.Sc in Microbiology in 1967, and PhD in Bacteriology in 1971 from the University of Bristol, UK. She spent six years as a post-doctoral research scientist in the Medical Research Council Dental Unit at the University of Bristol Dental School. In 1976 she moved to the University of Bradford, UK as Lecturer in Medical Sciences and in 1980 moved to University College London (UCL) as Senior Lecturer in Oral Microbiology in the Dental School. From 1992-2011 she was Senior Lecturer, Department of Medical Microbiology, later renamed as the Centre for Infectious Diseases and International Health, Division of Infection and Immunity, UCL. She has taught several generations of medical, dental and science undergraduate and postgraduate students. Her present position at UCL is Honorary Senior Lecturer, Division of Biosciences, Centre for The History of Medicine and Division of Infection and Immunity, Centre for Clinical Microbiology. She is a Fellow of the Royal Society for Tropical Medicine and Hygiene and member of several microbiological and professional societies. Initially her research interest was the microbial ecology of the human commensal oral flora. On transferring to the UCL Medical School she has worked primarily on the pathogenic mycobacteria. Her principal research expertise is in human pathogens, primarily *Mycobacterium tuberculosis* and *Mycobacterium leprae*, especially the detection and characterization of their DNA in archaeological material, using PCR. Her recent publications are on the molecular characterization of ancient *M. tuberculosis* and *M. leprae* and how this contributes to our understanding of the origin and evolution of pathogenic bacteria and their hosts.