Chapter 4
Molecular Detection of Past Pathogens

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Abstract Detection and characterisation of DNA is the most widely used approach for the study of past pathogens. This approach can be applied to various specimens, including environmental, vector and animal reservoir specimens as well as human corpses. Experimental data indicated that host-associated microbial DNA can survive for 20,000 years, and bacterial DNA preserved in permafrost specimens has been dated up to 1 million years. Current protocols targeted one pathogen at a time and universal 16S rDNA-based detection of bacteria have yielded ambiguous results. There is no universal detection of ancient virus so far. Major human pathogens, e.g. *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Yersinia pestis*, *Rickettsia prowazekii*, *Bartonella* spp. and Spanish influenza virus have been detected in suitable human specimens. Ancient *M. tuberculosis* and *Y. pestis* organisms have been genotyped, whereas the entire RNA genome of Spanish influenza virus was reconstituted for extensive studies. Metagenomic approaches based on high throughput pyrosequencing may help further resolve forthcoming issues. Interpretation of experimental data has to be based upon strict rules due to potential contamination of specimens.

4.1 Introduction

As a discipline, palaeomicrobiology (Zink et al. 2002) began in 1993 with the molecular detection of *Mycobacterium tuberculosis* DNA in an ancient human skeleton (Spigelman and Lemma 1993). This finding served to illustrate the importance of molecular biology techniques in the quest for pathogens, and microbes at large, in ancient specimens recovered from various human tissues, as well as from environmental samples of potential vectors and reservoirs of past pathogens

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(Drancourt et al. 2005). Indeed, with the exception of some enteric parasites (Bouchet et al. 2003) and rare human viruses, all past pathogens and microbes have been detected and studied thanks to the detection and characterisation of nucleic acids. Experimental data have now demonstrated that bacterial DNA can be detected in 20,000-year-old host specimens, and in up to several thousand-year-old environmental specimens preserved in permafrost (Willerslev et al. 2005). Likewise, Spanish influenza virus RNA has been extensively studied after its recovery from both formalin-preserved human lung tissue (Reid et al. 2000; Taubenberger et al. 1997) and permafrost-preserved human tissues (Reid et al. 2000).

The objectives of molecular detection of past pathogens include the diagnosis of past infectious diseases through the identification of specific molecular sequences in ancient remains; the elucidation of the epidemiology of past infectious diseases by reconstituting the temporal and geographical distribution of infected individuals, reservoirs and vectors; and the tracing of the genetic evolution of the microorganisms themselves through genotyping (Drancourt and Raoult 2005). Data from such studies benefit modern microbiology and studies of host–pathogen relationships. Refinements in molecular typing now allow researchers to study the genetic evolution of microorganisms and the timing of their introduction into human populations. Initial palaeomicrobiological studies used bone tissue, whereas later studies have progressed to using mummified tissues and dental pulp for analysis (Salo et al. 1994; Drancourt et al. 1998; Raoult et al. 2000) (Fig. 4.1). As for bone tissues, it was shown that both the gross and histological preservation were correlated with DNA survival (Haynes et al. 1970). Concomitantly, experimental standards for palaeomicrobiology have emerged to deal with the problems of contamination and the authenticity of data.

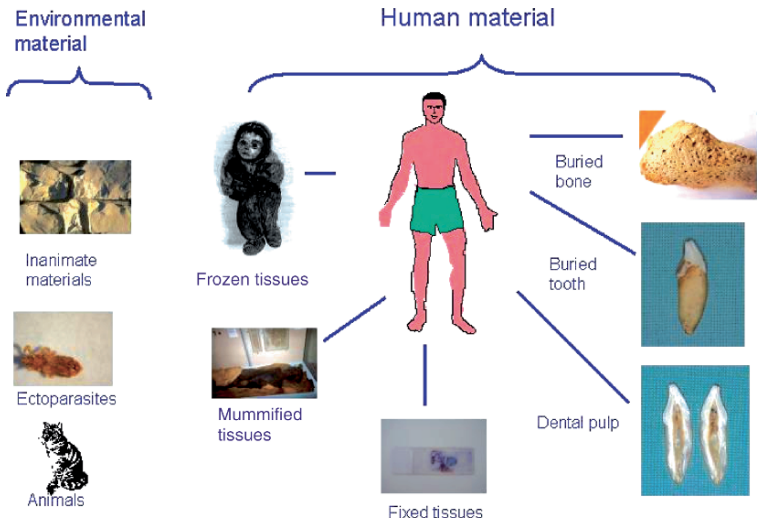

Fig. 4.1 Suitable source materials for amplification and sequencing of ancient microbe DNA

4.2 Protocols for the Molecular Detection of Past Pathogens

Detection and identification of pathogens in ancient human and environmental specimens relies mostly upon the molecular detection of specific nucleic acid sequences. A few studies have focussed on viral RNA for the detection of Spanish influenza virus (Reid et al. 2000; Taubenberger et al. 1997; Reid et al. 1999) but the vast majority of studies have targeted ancient bacterial and parasite DNA. While experimental protocols for DNA extraction and its amplification by polymerase chain reaction (PCR) have been empirical, a few systematic studies of experimental parameters now provide clear experimental guidelines for optimal DNA extraction and amplification from bone tissues (Rohland and Hofreiter 2007).

4.2.1 Ancient DNA Characteristics

Empirical observations made over the last 20 years indicated that ancient DNA has adverse characteristics when compared to modern DNA. The amino-acid racemisation ratio was shown to predict the preservation of ancient DNA (Poinar et al. 1996). Ancient DNA is broken into pieces of <500 bp (Lindahl 1993); consequently, PCR cannot be used to amplify large fragments in ancient specimens. In the case of ancient mammal DNA, this limitation has been circumvented by pre-treatment of the ancient DNA with reconstructive polymerisation (Golenberg et al. 1996) or enzymatic repair by the combined activities of DNA polymerase I and T4 DNA ligase (Pusch et al. 1998; Di et al. 2002). However, nothing has been published regarding the repair of ancient microbial DNA.

A second feature of ancient DNA is chemical modification, comprising both oxidisation and hydrolysis resulting in deamination of nucleotides (Hoss et al. 1996; Hofreiter et al. 2001). Such modifications have been implicated in cases of poor yields from PCR. It has been recently demonstrated that not all DNA polymerases amplify ancient DNA extracted from cave bear bone with the same efficiency (Rohland and Hofreiter 2007).

Third, numerous studies have demonstrated the presence of poorly characterised PCR inhibitors in ancient specimen extracts (Hoss et al. 1996; Hanni et al. 1995). The precise nature of these inhibitors, once correlated to the presence of a brown coloration of extracts (Hanni et al. 1995), is not known. Two strategies have been proposed to circumvent the presence of inhibitors: dilution of extracted specimens and the addition of bovine serum albumin (BSA). The effectiveness of both solutions has recently been demonstrated (Rohland and Hofreiter 2007).

4.2.2 Nucleic Acid Extraction

Since the initial demonstration that DNA can survive in mummified human tissues (Pääbo 1985), nucleic acid extraction from various types of specimens has been

Table 4.1 Adverse characteristics of ancient microbial DNA limiting PCR-based detection of past pathogens and proposed solutions. *PCR* Polymerase chain reaction, *BSA* bovine serum albumin

Characteristic	Consequence for PCR-based detection	Proposed solutions
Fragmentation < 500 bp	Amplification of small fragments only	Select PCR primers in order to amplify a fragment ≤ 300 bp DNA enzymatic repair using DNA polymerase I/T4 DNA ligase[a]
Chemical alterations	Poor PCR yield	Select appropriate *Taq* DNA polymerase
PCR inhibitors	Lack of PCR amplification	Run dilutions of extracted DNA Add BSA to PCR mix

[a] This technique has been published only for ancient eukaryotic DNA

achieved. Extraction can be achieved from conjunctive tissues that have been either frozen (Reid et al. 1999; Cano et al. 2000; Rhodes et al. 1998), mummified (Salo et al. 1994; Fornaciari et al. 2003) buried (Reid et al. 1999) (Table 4.1) or fixed (Taubenberger et al. 2005) . Extraction from bone tissues requires extensive decalcification using EDTA and mechanical grinding prior to DNA extraction. The same holds true for entire teeth. We proposed the use of dental pulp as a suitable specimen for the molecular detection of blood-borne organisms (Drancourt et al. 1998). Several protocols for the extraction of DNA from ancient tissues have been proposed, but the comparative performance of these various protocols has been evaluated only recently (Rohland and Hofreiter 2007).

4.2.3 Amplification, Cloning and Sequencing

All studies dealing with ancient microbial DNA use a PCR amplification step before nucleotide sequencing. Various PCR protocols have been developed, including one-step conventional PCR in most studies, nested and hemi-nested PCR and, rarely, real-time PCR. The addition of either BSA or a related protein in the PCR cocktail had been advocated in order to prevent PCR inhibition (Rohland and Hofreiter 2007). This empirical observation has recently been verified (Rohland and Hofreiter 2007). The exact nature of the PCR inhibitors in ancient specimens has not been elucidated, and the proposed correlation of the brown colour of the extraction product with PCR inhibition (Hanni et al. 1995) has not been confirmed (Drancourt et al. 1998). In most studies, PCR-amplified fragments are cloned before being sequenced. So far, the conventional Sanger sequencing method has been applied using capillary automatic sequencers.

4.3 Contamination of Ancient Specimens

Micro-organisms from the burial site can contaminate specimens before laboratory analyses, whereas laboratory micro-organisms and their DNA can contaminate specimens during laboratory analyses. Some PCR mix reagents, including PCR primers, polymerases and water used to complement reaction volumes, have been shown to be contaminated by bacterial DNA. In the detection of past bacteria, the contamination threat is particularly great when using a universal approach such as 16S rDNA-based PCR (Gilbert et al. 2004; Zink et al. 2000; Cano et al. 2000). Specific molecular targets carry a smaller risk. The specificity of detection has been shown by analysis of environmental samples in parallel with buried specimens (Papagrigorakis et al. 2006). The use of naturally protected specimens, such as dental pulp, might also limit the risk of external contamination (Drancourt et al. 1998).

4.4 Strategies to Obtain Reliable Data

Several protocols can be used to limit the risk of contamination in the laboratory (Fig. 4.2, Table 4.2). The external cleansing of bone using filtered compressed air and sterile distilled water, scraping the external surface, and irradiation with 254-nm ultraviolet (UV) light have all been advocated (Ou et al. 1991). For the manipulation of ancient teeth, encasing the specimen in sterile resin has been proposed (Gilbert et al. 2003). All PCR-based experiments should be carried out in designated one-way PCR suites with appropriate ventilation. Primer optimisation for PCR should be carried out in a separate building from the one in which the ancient material is handled, and

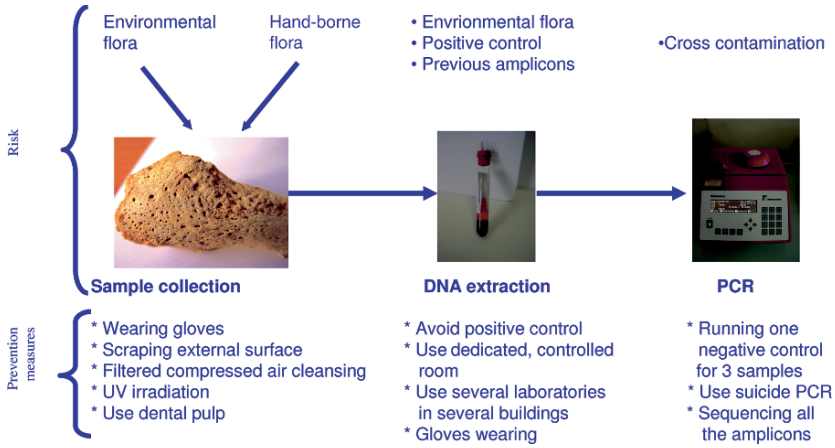

Fig. 4.2 Prevention of bacterial and molecular contamination in palaeomicrobiology

Table 4.2 Prevention of specimen contamination in ancient microbial DNA studies

Source of contamination	Proposed solutions
Burial site:	
Environmental flora	External surface scraping, sterile water and filtered compressed air cleansing
	UV irradiation
	Using dental pulp
Hand-borne flora	Wearing gloves for specimen manipulation
Laboratory:	
Environmental flora	Wearing gloves for specimen manipulation
Hand-borne flora	Respect of strict protocols
PCR reagents	Use of dedicated, controlled rooms
Previous experiments	Suicide PCR
Cross-contamination	No positive control
	Negative controls
	Amplicon sequencing

PCR and post-PCR experiments should be performed in a separate room using disposable equipment and freshly prepared reagents that have been irradiated with UV light. It has been also advocated that ancient DNA experiments be performed without using a positive control. Alternatively, mock positive controls and DNA from different, related species can be used. Furthermore, we developed "suicide PCR" reactions, which target a new genomic region by using a new PCR primer pair in every new experiment, to prevent vertical contamination from previous amplifications (Raoult et al. 2000). The introduction of numerous negative controls helps monitor any carry-over source of contamination. Material collected from unaffected individuals are also of value; for example, lesion-free bones collected from fossilised *Canis* and *Equus* species have been used as controls for the molecular detection of *M. tuberculosis* DNA in extinct bison (Rothschild et al. 2001).

As pathogens are not ubiquitous organisms, the first sequence achieved in a laboratory is reliable if the pathogen and its DNA have never been manipulated in that laboratory. Therefore, standardisation of PCR protocols must be carried out in a laboratory different from the one where the ancient DNA is handled. Likewise, DNA from ancient specimens must be extracted in a laboratory where the targeted pathogens have never been manipulated. We optimised this approach by performing these different experimental steps in laboratories located in different campus buildings (Drancourt et al. 2004). Also, we designed suicide PCR in order to prevent intra-laboratory contamination resulting from previous experiments (Raoult et al. 2000). Suicide PCR avoids use of positive controls and uses a new PCR primer pair targeting a different genomic region for every new experiment (Raoult et al. 2000). Alternatively, PCR targetting a hypervariable genomic region could be used in order to demonstrate the presence of an original sequence of the pathogen in the ancient specimen.

4.5 Interpretation of Data

Strict adherence to the rules for the prevention of contamination is a first step towards ensuring the authenticity of ancient microbial isolates. Absence of any amplicon in negative controls is strictly required. The recovery of an original sequence indicates that laboratory contamination has not occurred and is good evidence for authenticity. The original sequence must be shown in several clones. Chemical modifications of ancient DNA can result in "jumping PCR" – template switching during PCR and C→T and G→A substitutions. The sequencing of multiple clones derived from more than one independent amplification has been advocated to reduce the risk of obtaining incorrect DNA sequences (Hoss et al. 1996; Spencer and Howe 2004). However, there is no evidence of "spontaneous" mutation in ancient DNA (Serre et al. 2004).

Phylogenetic analyses of the gene sequence from the ancient microorganism can confirm its antiquity; for example, phylogenetic analyses of a *Bacillus* sp. that was once claimed to be 250 million years old showed that it was in fact a modern contaminant (Vreeland et al. 2000; Nickle et al. 2002). The reproducibility of results using different specimens collected from the same individual is another a criterion. Also, the demonstration of two unrelated sequences that identify the same pathogen in the same specimen further increases the specificity of the identification.

4.6 Molecular Detection of Past Pathogens: Current Data

Most published data deal with the detection and molecular characterisation of ancient bacteria, while fewer studies have examined past viruses and parasites. The most significant data are presented in Table 4.3. To complement the molecular detection and identification of past pathogens, some ancient bacteria have been genotyped. In the case of the *M. tuberculosis* complex, ancient mycobacteria were genotyped by sequencing the phospholipase-C *mtp*40 gene, a *Mycobacterium tuberculosis*-specific region, another *Mycobacterium bovis*-specific fragment and the *oxy*R pseudogene (Pääbo 1985). This work demonstrated that medieval mycobacteria were more closely related to modern *Mycobacterium tuberculosis* than to *Mycobacterium bovis*. Similar conclusions were obtained from a spoligotyping analysis of 12 *Mycobacterium tuberculosis* strains that were characterised among Egyptian mummies dating from 2050 to 500 B.C. (Zink et al. 2003). Spoligotypes obtained from mycobacterial DNA from an extinct bison demonstrated that it was more closely related to the *Mycobacterium tuberculosis* / *Mycobacterium africanum* group than it was to *M. bovis* (Rothschild et al. 2001). These data indicated that the theory that *Mycobacterium tuberculosis* had evolved from *M. bovis* by specific adaptation to the human host was not in fact the case (Stead et al. 1995).

In our laboratory, using multispacer sequence typing (MST), we have successfully genotyped *Yersinia pestis* in individuals suspected to have died from the Justinian

Table 4.3 Current data in palaeomicrobiology. *BP* before present (years)

Bacteria	Source	Specimen, body site	Conservation	Date	Reference
Mycobacterium tuberculosis	Bison	Metacarpal	Buried	17,000 BP	Rothschild et al. 2001
	Human	Lung, lymph node	Mummified	1,000 BP	Salo et al. 1994
	Human	Bone	Mummified	5,400 BP	Crubezy et al. 1998
	Human	Metacarpal, lumbar vertebrae	Buried	Medieval	Taylor et al. 1999
	Human	Rib	Buried	Medieval	Mays et al. 2002
	Human	Vertebrae	Buried	1,000 BP	Arriaza et al. 1995
	Human	Mandible	Buried	1400–1800 A.D.	Hass et al. 2000a
	Human	Vertebrae, femur, ankle, rib, pleura	Buried	Seventh–eighth centuries; seventeenth century	Hass et al. 2000a
	Human	Lung pleura	Buried	600 A.D.	Donoghue et al. 1998
	Human	Bone	Buried	1,000 BP	Gernaey et al. 2001
	Human	Vertebrae, rib	Buried	400–230 B.C.	Mays and Taylor 2003
	Human	Bone, soft tissues	Mummified	2050–500 B.C.	Hanni et al. 1995
	Human	Bone	Buried		Spigelman and Lemma 1993
	Human	Lungs, pleura, abdomen, ribs, hair, teeth	Mummified	Eighteenth–nineteenth centuries	Fletcher et al. 2003
	Human	Wrist, lumbar vertebrae	Buried	Fourteenth–sixteenth centuries	Taylor et al. 1996
Mycobacterium leprae	Human	Foot bones	Buried	Twelfth century	Montiel et al. 2003
	Human	Metacarpals	Buried	300–600 A.D.	Spigelman and Donoghue 2001
	Human	Skulls	Buried	1400–1800 A.D.	Donoghue et al. 2001
	Human	Hard palate, skull	Buried	1400–1800 A.D.; tenth century	Hass et al. 2000a
Enteric bacteria	Mastodon	Bowel	Frozen	12,000 BP	Rhodes et al. 1998
	Human	Metatarse	Mummified	1400 B.C.	Zink et al. 2000

	Human	Upper gut content	Preserved in bog	300 B.C.	Fricker et al. 1997
Treponema pallidum	Human	Bone	Buried	240 BP	Kolman et al. 1999
Borrelia burgdorferi	Ticks		Dry	1884	Matuschka et al. 1996
	Rodents		Dry	Nineteenth century	Marshall et al. 1994
Spirochetes	Termite	Instestinal tissue	Amber	Miocene	Wier et al. 2002
Bartonella quintana	Human	Dental pulp	Buried	4,000 BP	Drancourt et al. 2005
Bartonella henselae	Cat	Dental pulp	Buried	Thirteenth–eighteenth centuries	La et al. 2004
	Human	Dental pulp	Buried	Fifth–fourteenth centuries	Drancourt et al. 2004
	Human	Dental pulp	Buried	1590–1722	Drancourt et al. 1998
	Human	Dental pulp	Buried	1348	Raoult et al. 2000
Rickettsia prowazekii	Human	Dental pulp	Buried	1812	Raoult et al. 2006
Mixed flora	Human	Skin/muscle	Frozen	Neolithic	Rollo et al. 2000
Mixed flora	Human	Colon	Frozen	Neolithic	Cano et al. 2000
Parasites					
Ascaris lumbricoïdes	Human	Coprolites	Buried	Middle-Ages	Loreille et al. 2001
Plasmodium falciparum	Human	Bone	Burried	1,500 BP	Taylor et al. 1997
Trypanosoma cruzi	Human	Human, visceral tissue	Mummified	4,000 BP	Guhl et al. 1997
	Human	Heart, lung, liver, kidney, ileum; colon, muscle, brain	Mummified	9,000 BP	Aufderheide et al. 2004
	Human	Bone	Mummified	4,000 BP	Zink et al. 2006
Enterobius vermicularis	Human	Corpolites	Buried		Loreille et al. 2001
Viruses					
HTLV-I	Human	Bone		1,500 BP	Li et al. 1999
HPV	Human	Skin	Mummified	Sixteenth century	Fornaciari et al. 2003
Influenza virus	Human	Lung	Fixed	1918	Taubenberger et al. 1997
			Frozen	1918	Reid et al. 2000

plague (Drancourt et al. 2004). After comparison of the two *Y. pestis* genome sequences available in GenBank, we found that some intergenic spacer sequences were highly variable, and we amplified six of these sequences from the ancient specimens. Sequence analyses showed that the sequences obtained were original sequences owing to the presence of point mutations. These mutations were consistently found in several clones, therefore confirming that they were not merely caused by misincorporation of nucleotides by *Taq* polymerase. *Y. pestis* has been subdivided into three biovars on the basis of their ability to ferment glycerol and to reduce nitrate. On the basis of their current geographical niche, and on historical records that indicated the geographical origin of the pandemics, it was speculated that each biovar caused a specific pandemic (Devignat 1954). MST data demonstrated that the genotype involved in all three pandemics was associated with the Orientalis biovar, a result recently confirmed by demonstration of a specific deletion in the *glp*D gene (Drancourt et al. 2007).

4.7 Future Research

The detection of pathogens in their ancient reservoirs, and of vectors, will be a key factor in achieving the goal of a global epidemiology scheme for every transmissible infectious disease. Such detection will benefit from improved collaboration between palaeozoologists, specialists in ancient ectoparasites and palaeomicrobiologists. Specific issues include the correct collection and identification of buried animals and ectoparasites. With regards to human remains and the remains of other mammals, in our opinion, the broad use of dental pulp could help resolve the aetiology of ancient bloodborne infections, although universal protocols are still required.

The application of the universal 16S rDNA-based detection and identification of bacteria to palaeomicrobiology has been limited by contamination of the ancient material. However, this powerful molecular tool will be invaluable in the study of the nature and epidemiology of unpredicted pathogens. The aetiology of numerous past epidemics remains unknown, despite testing for the presence of one or more bacterial pathogens. Tracing any bacterial pathogen within the remains of this past population could help resolve the question of the aetiology of some mysterious epidemics. Given the small amount of material available in the majority of these cases, testing for all bacterial pathogens simultaneously would be helpful. Studies must be performed to develop a protocol of universal amplification and sequencing that is adapted to ancient bacterial DNA.

Metagenomic analysis of total DNA extracted from ancient specimens is a promising field of research. It relies on the high throughput sequencing made possible by the new generation of pyrosequencers. This new approach has been successfully applied to the study of complex modern flora, and to that of ancient mammoth tissue (Poinar et al. 2006). It may resolve the quest for universal detection, not only of bacteria but also of viruses, in ancient specimens.

Genotyping will create the necessary bridge between the detection of microbial DNA in ancient environmental and human specimens and modern microbiology. The availability of a large database of complete microbial genome sequences has already prompted the establishment of suicide PCR and new genotyping methods for past microorganisms, including spoligotyping of *M. tuberculosis* (Zink et al. 2003) and MST of *Y. pestis* (Drancourt et al. 2004). Such efforts should be continued.

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