Evaluating bacterial pathogen DNA preservation in museum osteological collections

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Reports of bacterial pathogen DNA sequences obtained from archaeological bone specimens raise the possibility of greatly improving our understanding of the history of infectious diseases. However, the survival of pathogen DNA over long time periods is poorly characterized, and scepticism remains about the reliability of these data.

In order to explore the survival of bacterial pathogen DNA in bone specimens, we analysed samples from 59 eighteenth and twentieth century individuals known to have been infected with either *Mycobacterium tuberculosis* or *Treponema pallidum*. No reproducible evidence of surviving pathogen DNA was obtained, despite the use of extraction and PCR-amplification methods determined to be highly sensitive. These data suggest that previous studies need to be interpreted with caution, and we propose that a much greater emphasis is placed on understanding how pathogen DNA survives in archaeological material, and how its presence can be properly verified and used.

Keywords: ancient DNA; tuberculosis; syphilis; bone; evolution; medicine

1. INTRODUCTION

The study of pathogen DNA recovered from archaeological and archival material has potential to contribute to a wide variety of questions about past human health. It has been proposed as a means of confirming diagnoses from skeletal material, to allow the determination of the disease state, when characteristic osseous lesions are absent or not fully diagnostic, and to enable us to facilitate a deeper understanding evolution of pathogens using phylogenetic and population genetic analyses (Zink *et al.* 2002; Donoghue *et al.* 2004). Yet, while there have been some notable advances in this area of research, such as the identification of 1918 influenza virus (Taubenberger *et al.* 1997; Stevens *et al.* 2004), this field remains as one of the more contentious in ancient DNA research (Kolman *et al.* 1999; Drancourt & Raoult 2004; Gilbert *et al.* 2004a,b; Prentice *et al.* 2004; Bouwman & Brown 2005; Weichmann & Grupe 2005).

Partly, this is because ancient pathogen analyses have rejected technical standards employed in other ancient DNA work: high profile studies have sometimes incorporated high copy number templates as PCR positive controls (Salo *et al.* 1994; Fricker *et al.* 1997); the presence of host mitochondrial or nuclear DNA is not demonstrated (see, however, Taylor 1996; Barnes *et al.* 2000; Zink *et al.* 2001a); replication of results is not reported, or differences are found between replicates (Rothschild *et al.* 2001; Taylor *et al.* 2003); few studies conduct cloning of PCR products or quantification of template DNA (see, however, Zink *et al.* 2001b); and identifications are made on the basis of PCR success or RFLP patterns rather than sequencing (Guhl *et al.* 1999; Mays *et al.* 2002).

Recent reports have begun to incorporate these standards (Donoghue *et al.* 2005; Taylor *et al.* 2005), and demonstrate an increasing sophistication in approach.

However, concerns about the veracity of ancient pathogen DNA results are not only based on differences in technical method, but also recognition of fundamental taphonomic and conceptual issues inherent in the use of ancient DNA.

Our understanding of the effects of post-mortem change to DNA, while far from complete, has advanced significantly in the past two decades. The importance of this knowledge has become obvious: understanding the relationship between specimen history and DNA preservation provides a basis for establishing in which specimens DNA is unlikely to survive, and positive results are, therefore, due to external contamination (Collins *et al.* 2002; Smith *et al.* 2003), and when DNA is likely to be damaged, leading to inaccuracies in resulting sequence by PCR-induced replacement of certain bases (Hansen *et al.* 2001; Hofreiter *et al.* 2001a; Gilbert *et al.* 2003a,b).

The general issues of preservation, contamination and decay have more specific counterparts in the study of ancient pathogen DNA, and their solution remains a prerequisite for utilizing the data in any meaningful way. The principle unknown is how sufficient pathogen DNA, present at low concentration in the living host, survives in an archaeological specimen. This problem stems in part from a lack of knowledge of the quantity of pathogen DNA liable to be present in bone, and in part from the more general question of where and how the ancient biomolecules in bone are preserved.

A second problem comes in differentiating contaminant sequences from those endogenous to the sample. There are many ways of mitigating PCR contamination; however, none of them can be considered absolutely effective—particularly, in cases, where the contaminant has entered the sample prior to laboratory extraction. We are, therefore, dependent on the detection of PCR contamination *after* analysis has been conducted. Nearly all methods of doing so rely on the presence of sequence

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differences between the contaminant and the target sequence. However, most of the bacterial pathogens studied in archaeological projects have very limited sequence diversity between strains, with typically one or no polymorphic sites per amplified fragment. With low numbers of starting template molecules, and site-directed damage, differentiating true sequences from a damaged template or contamination becomes almost impossible (Gilbert *et al.* 2003b).

This study was designed to establish baseline data on the survival of ancient pathogen DNA and the extent to which post-mortem damage might affect the reliability of data, employing a more explicitly experimental approach than previous work. To do so we analysed individuals from two collections of human remains for whom cause of death was known to be either tuberculosis (TB) or syphilis, the two principal bacterial infections for which DNA recovery has been investigated in archaeological material. PCR-based assays, derived from the ancient pathogen literature, were developed to detect *Mycobacterium tuberculosis* and *Treponema pallidum*, the respective causative agents of these diseases.

2. MATERIAL AND METHODS

The majority of the 62 samples (from 59 individuals) came from two well-documented collections of human remains: the Hamann–Todd (HTH) collection housed at the Museum of Natural History in Cleveland, Ohio and the Hunterian collection of the Royal College of Surgeons of England (RCS). The two collections were assembled for quite different reasons; the HTH material is a general osteological collection largely made up of unclaimed cadavers dating 1911–1938, with cause of death known from medical records. The collection is extensive ($n \approx 3000$), with around 20% of individuals recorded as having died from TB, and a similar proportion from syphilis. The characteristic skeletal lesions associated with these infections are, however, exceptionally rare in this material. The Hunterian collection, dating from the latter half of the eighteenth century, contains approximately 3500 natural history specimens. TB- and syphilis-infected specimens show classic osteological modifications of the relevant disease. Finally, a single specimen from the Prague Museum of Medicine was included in the analysis (see table 1).

These collections share several advantages over archaeological material for the purposes of this project—in addition to recent date of death, they have never been subjected to the burial environment, and medical diagnoses of infection are available. As with most archaeological material, we lack information about the post-mortem handling and preparation of the material. It seems likely that, at least for the HTH material, some form of sterilization would have taken place. However, even the most stringent cleaning procedures used at present, including repeated and lengthy boiling steps and chemical treatments, still allow the recovery of DNA from bone (e.g. Faerman *et al.* 2000; Arismendi *et al.* 2004). Exposure to high temperature is an element of many DNA handling procedures, including PCR and DNA preparation; the TB positive control DNA used in this study, for example, was prepared by boiling cultured cells.

The complexities of manipulating ancient DNA are well documented (Paabo *et al.* 1989; Kolman & Tuross 2000; Hofreiter *et al.* 2001b), and all attempts were made

to ensure that contamination of the material was avoided. Specifically, all pre-amplification laboratory work was conducted in a dedicated facility, physically isolated from the post-PCR areas, and work surfaces frequently cleaned with 10% sodium hypochlorite solution and irradiated overnight with UV light; disposable plastic items were used whenever possible, non-disposal items were baked at 200 °C overnight or washed with hypochlorite; solutions were bought in pre-made, and all work was conducted while wearing appropriate protective garments. Contamination was monitored through the use of multiple blanks. Modern *M. tuberculosis* and *T. pallidum* genomic DNA was used to establish PCR-assay efficiency, but these experiments did not overlap with work on the museum samples.

DNA extraction was as previously described (Barnes *et al.* 2002), modified by scaling down to 2 ml total volume. The solvent-based approach chosen has been successfully used to recover DNA in studies on both modern and ancient TB (Kox *et al.* 1994; Hashimoto *et al.* 1995; Baron *et al.* 1996). PCR amplifications used a range of primers (table 2), some designed for this project, others chosen from the ancient DNA literature on the basis of high sensitivity, frequent application in this context, or because they amplify a polymorphic region (Taylor *et al.* 2003). Platinum *Taq* Hifi (Invitrogen) was used in all amplifications of museum extracts, and reaction conditions were as previously described (Barnes *et al.* 2002). Cycling conditions were chosen after preliminary trials to provide optimal sensitivity and stringency. For those primers drawn from the existing literature, the conditions used are in good agreement with those previously published. Nested PCR was not conducted for the IS6110 primer set, as it was already highly sensitive with only a single primer pair, and it is not possible to avoid PCR product contamination after the first round of amplification. For each primer pair used, template standards of known concentration were generated and quantified using PicoGreen (Molecular Probes) after removal of unincorporated primers and nucleotides. Primer sensitivity was then determined by amplification of serial dilutions of these standards under the conditions employed above.

All PCR primer pairs appropriate to the infection carried by the sample were used on at least three occasions for each sample.

At first, PCR products of approximately the correct size were directly sequenced with ABI Big Dye Terminator chemistry and resolved on an ABI 3100 automated sequencer. However, most products generated comprise a mixture of sequences, and so were cloned into the TOPO TA (Invitrogen) vector, and colonies were PCR-screened. Sequences obtained were used as the query for BLAST searching of the NCBI database.

In order to establish that DNA extraction removed compounds that could inhibit PCR amplification, 1 µl volume was taken from a subset of museum samples ($n=8$; see table 1), representing individuals from both collections. These replaced an equivalent volume of water in the amplification of a cervid DNA template, known by dilution experiments to be at the limit of detection for the PCR system used. The PCR-inhibitory effect of the museum DNA extracts was assessed by comparison of the cervid DNA amplification, with and without addition of the museum extract.

Table 1. Samples used in this study.

specimen	element sampled	pathology	age—race—sex
<i>Hunterian collection</i>			
P715a	calvaria	syphilis	—
P715b	calvaria	syphilis	—
P717	calvaria	syphilis	—
P718	calvaria	syphilis	—
P719	calvaria	syphilis	—
P720	calvaria	syphilis	—
P731	femur	syphilis	—
P732	femur	syphilis	—
P733	tibia	syphilis	—
P746	fibula	syphilis	—
P885	rib/spine	tuberculosis	—
P888	vertebra	tuberculosis	—
P890	vertebra	tuberculosis	—
P891	vertebra	tuberculosis	—
P897	pelvis/femur	tuberculosis	—
<i>Hamann-Todd collection</i>			
HTH0018	rib	pulmonary tuberculosis	52WM
HTH0027	rib	pulmonary tuberculosis	45BM
HTH0031	rib	pulmonary tuberculosis	47BM
HTH0036	rib	pulmonary tuberculosis	47WM
HTH0038	rib	tuberculosis	38BM
HTH0153	clavicle	tuberculosis	27WM
HTH0155	rib	tuberculosis	46WM
HTH0218	rib	tuberculosis	50WM
HTH0238	rib	pulmonary tuberculosis	30WM
HTH0241	rib	pulmonary tuberculosis	40BM
HTH0258	(1) rib	pulmonary tuberculosis	56WM
HTH0258	(2) femur		
HTH0262	rib	pulmonary tuberculosis	35WM
HTH0285	rib	pulmonary tuberculosis	45WM
HTH0296	rib	tuberculosis	48WM
HTH0448	rib	pulmonary tuberculosis	31BM
HTH0470	rib	pulmonary tuberculosis	35WM
HTH0448	rib	pulmonary tuberculosis	31BM
HTH0470	rib	pulmonary tuberculosis	35WM
HTH0475	rib	tuberculosis	37BM
HTH0638	cranium	syphilis	33BM
HTH0641	(1) rib	tuberculosis	53WM
HTH0641	(2) femur	—	—
HTH0643	femur	tuberculosis	26WM
HTH0646	femur	tuberculosis	40BM
HTH0647	(1) radius	tuberculosis	38WM
HTH0647	(2) rib		
HTH1014	rib	pulmonary tuberculosis	22BM
HTH1084	sternum	pulmonary tuberculosis	24BM
HTH1090	cranium	syphilis	47WM

(Continued.)

Table 1. (Continued.)

specimen	element sampled	pathology	age—race—sex
HTH1091	rib	pulmonary tuberculosis	25WM
HTH1116	rib	pulmonary tuberculosis	31BM
HTH1178	rib end fragment	tuberculosis	—
HTH1464	cranium	syphilis	60WM
HTH1480	rib	tuberculosis	50BM
HTH1737	rib	tuberculosis	58WM
HTH1767	cranium	syphilis	77WF
HTH1851	cranium	syphilis	33BM
HTH2176	cranium	syphilis	45WM
HTH2177	cranium	syphilis	70BM
HTH2319	cranium	syphilis	75BM
HTH2535	rib	pulmonary tuberculosis	47WM
HTH2588	cranium	syphilis	50WM
HTH2793	cranium	syphilis	39BM
HTH0285	tooth	pulmonary tuberculosis	45WM
HTH2874	femur	syphilis	57BM
HTH2944	tibia	syphilis	33BM
HTH3011	cranium	syphilis	35BM
<i>Prague Museum of Medicine collection</i>			
ANM2010	osseous gumma	syphilis	—

3. RESULTS

Across all amplifications, DNA fragments of approximately the anticipated size were recovered on 15 occasions. Database identifications of the sequences obtained fell into three classes:

- some homology to a previously described sequence, but not the target;
- little or no homology to any previously described sequence;
- matching the targeted sequence.

Four amplifications with *rpoB* primers yielded products that were homogenous enough to be sequenced directly. Two products (specimens HTH0038 and 0155) matched portions of the 16SrRNA of *Propionibacterium acnes* (99% identity); a third (HTH0238) had distant homology (90%) to *Rhodococcus equi*, *Corynebacterium renale* and various environmental mycobacteria, including *Mycobacterium tokaiense*, *murale*, *aurimucosum*, *pilosum* and *diernhoferi*. A fourth sample (HTH0116) had distant homology with a different set of mycobacteria, including *Mycobacterium obuense* (92%) and *Mycobacterium intracellulare*, *gadium*, *fallax*, *vanbaalenii*, *senegalense*, *farcinogenes*, *fallax*, *chubuense* (all 90%).

Two clones from an amplification of sample HTH0470 with IS6110 primers gave a sequence with a close (98%) homology to the urease G gene of *Klebsiella aerogenes*.

Only a single PCR product was generated in attempts to amplify *T. pallidum* DNA, from sample ANM2010. Cloning identified this amplicon as derived from at least three different templates, one with 87% homology to *Staphylococcus aureus*, and the others non-identifiable.

Table 2. PCR conditions and primer sensitivity for this study. (TPP15-L171 (GCGTTCTGCCCTTTTGACGTTG)/H86 (CCGACTGCTCAGCCCACT GTCTT); katG-F(CGGTCCCTGCGGTCAGCC)/R(TCGCTACCACGGAACGACG AC); gyrA-F(ACCGCAGCCACGC CAAGTC)/R(GGTAGCGCAGCGACCAGGG). Limit of detection for Mays *et al.* 2001 derived from the paper using a *M. tuberculosis* genome size of 4 411 532 bases (Cole *et al.* 1998). Limits are given as copy numbers between which the PCR ceased to work. NA, not assessed.)

primer pair (target species)	anneal temperature (°C)	[Mg ⁺⁺] in PCR (mM)	size (bp)	limit of detection (copies)		
				this study	Mays <i>et al.</i> (2001)	reference
DR a/b (<i>M. tuberculosis</i>)	55	1	ca 85	5–25	NA	Kamerbeek <i>et al.</i> (1997)
TPP15-L171/H86 (<i>T. pallidum</i>)	55	2	123	80–410	NA	this study
CR 16209/16356 (<i>H. sapiens</i>)	56	2	184	2–10	NA	Handt <i>et al.</i> (1996)
L243/H123 (<i>T. pallidum</i>)	60	2	120	3–17	NA	Kolman <i>et al.</i> (1999)
rpoB F/R (<i>M. tuberculosis</i>)	62	2	157	3–13	3.3–33	Telenti <i>et al.</i> (1993)
oxyR F/R (<i>M. tuberculosis</i>)	62	2	150	14–67	33–330	Taylor <i>et al.</i> (1999)
gyrA F/R (<i>M. tuberculosis</i>)	62	2	124	4–17	NA	this study
katG F/R (<i>M. tuberculosis</i>)	63	2	139	15–73	NA	this study
IS6110-3F/4R (<i>M. tuberculosis</i>)	65	2	92	5–22	NA	Taylor <i>et al.</i> (1996)
mtp40 F/R (<i>M. tuberculosis</i>)	66	2	152	3–14	33–330	Taylor <i>et al.</i> (1999)

In class (ii), non-identifiable sequences were obtained using the katG primers from clones derived from amplification of eight samples (P888, 891, 987 and HTH0238, 0258, 0262, 0285, 0641).

Only a single example of class (iii), a matching sequence, was identified from these samples, an IS6110 amplification on sample RCS-P888. However, as this result was not reproducible in three further attempts to amplify the sample with these primers, it has been discounted as an example of contamination, presumably arising as a result of primer optimization prior to analysis of the ancient material.

4. DISCUSSION

Possible reasons for the differences between these results and those typically published are outlined in table 3 and addressed below.

(1) In order to establish that any DNA was still present in the samples, a 184 bp fragment of the human hypervariable mitochondrial control region was amplified and cloned from a subset ($n=9$) of HTH samples (table 1). IB, who conducted all handling of the material from the sampling stage onward, possesses a typically European haplotype (M) with an unusual polymorphism (an insertion between 16259 and 16262), which is sufficiently rare to have not been previously reported (Wallace & Lott 2004). Thus, in order to maximize the likelihood of sequence difference between the samples and IB, and thereby identify lab contamination, samples used in this exercise were identified as ‘black’ (as opposed to ‘white’) in the HTH archives.

Sequences were generated from five to ten clones for each sample (figure 1). To summarize: no PCR amplification was obtained from one sample; a single sequence, not attributable to IB, was found in two samples; a single sequence, identical to IB, was identified in one sample. In the remaining five samples, both the lab contaminant and 1–2 other sequences were identified. Where two non-IB sequences were identified from the same sample, the differences between them were sufficiently slight

(2–4 transitions) that they could be attributed to template damage (Gilbert *et al.* 2003a). A range of results, including template damage and contamination, is common in amplifications of degraded human material (e.g. Handt *et al.* 1994; Krings *et al.* 1997; Gilbert *et al.* 2003b). Thus, while post-mortem treatment of the specimens may have reduced DNA yields, it appears that host DNA survives in the material.

(2) It may be that, despite a known history of infection, bacteria are absent from the fragment of bone sampled. Specifically, the bacterial load might be heterogeneously distributed within the sample, either spatially (in different skeletal elements of the host body), or temporally (perhaps becoming lower just prior to death of the host). Additional possibilities include the sampling location being too far from a lesion (on the assumption that the bacterial load is only high near the point of skeletal remodelling) or too close (as the lesion represents only a former focus of destruction, and the bacteria are now elsewhere in the bone).

The clinical data required to assess these hypotheses are not available for the pathogens studied here, and given that they would require multiple peri- and post-mortem bone samples from individuals infected with potentially curable diseases, they are unlikely to become available. However, a survey of the extensive ancient TB literature shows that while most positive identifications come from specimens with some sort of skeletal lesion, many of these samples are not from locations proximal to a lesion (e.g. Taylor *et al.* 1996; Haas *et al.* 2000; Donoghue *et al.* 2005). On this evidence, we propose that the location of sampling is not critical, and that the recovered pathogen DNA must originally have been in the blood stream.

(3) It is possible that the bacterial load in the individuals sampled here was too low at death to allow subsequent successful PCR amplification. It is not clear, however, why the individuals tested here should have died with a substantially lower concentration of bacteria than those from other locations and time periods, where detection has been successful, especially in light of the

Table 3. Summary of possible explanations for results. (See §4 for discussion.)

possible problem	reason
no DNA in samples	DNA preservation poor (1)
host DNA in samples, no pathogen DNA was actually present in samples	absence of pathogen in the fragment of bone sampled (2)
host DNA in samples, pathogen was present, but is now too degraded to be recovered	due to: (i) low initial number of pathogen cells (3) (ii) preferential degradation of pathogen DNA (4)
DNA in samples, DNA not extracted	poor extraction technique (5)
DNA in samples, pathogen DNA not extracted	inappropriate extraction technique (6)
pathogen DNA not amplified, but present in extracts	(i) primers do not work (7) (ii) inhibition of PCR (8)

very high success rates given in published papers. In those studies working with relatively larger numbers ($n > 10$) of archaeological samples, detection rates are between 55 and 75% for samples with some prior evidence for TB (Haas *et al.* 2000; Zink *et al.* 2001a; Fletcher *et al.* 2003; Zink *et al.* 2003).

(4) While bacterial cells are generally more robust than those of humans, their nucleic acids are at a disadvantage with regard to long-term survival, as they are not integrated within bone structure in the way that human DNA is. It could, therefore, be argued that bacterial pathogen DNA is less likely to survive than that of the host. *Treponema pallidum* is at a particular disadvantage here, as it is found only in soft tissues and blood, and has a weak cell wall. *Mycobacterium tuberculosis*, on the other hand, is known to be sequestered by the immune system and contained within calcified lesions. Furthermore, it has been suggested (Zink *et al.* 2002; Donoghue *et al.* 2004) that the resistant mycolic acid component of the *M. tuberculosis* cell wall offers an explanation for its apparently enhanced survival, as these molecules are resistant to chemical and physical attack (Barry *et al.* 1998). However, the persistence of *M. tuberculosis* in the burial environment is not supported empirically. While DNA from members of the Mycobacteriaceae has been recovered from frozen soil of up to 3–400 000 years of age (Willerslev *et al.* 2004), they do not demonstrate any advantage over other Actinobacteria, which survive equally well. Under more temperate conditions, *Mycobacterium bovis* has been cultured from spiked soils and tissue samples that have been environmentally exposed for one to two months (Duffield & Young 1985; Tanner & Michel 1999). It may be that the pathogenic mycobacteria enter into an anabiotic state under these conditions, and that PCR, rather than culture, is necessary for detection. Further work is needed to reduce conjecture in this area.

(5) We can reject the possibility of failed extraction on the grounds that (i) the technique allows the recovery of host DNA from the samples, (ii) DNA sequences presumably derived from environmental bacteria are recovered from the samples and (iii) because this extraction technique, or a related version, has been used in a wide variety of published studies, including samples with marginal survival of bacterial, fungal and vertebrate DNA (Baron *et al.* 1996; Barnes *et al.* 2002; Bunce *et al.* 2004; Shapiro *et al.* 2004; Willerslev *et al.* 2004).

(6) Could the extraction method used in this study differentially recovered host but not pathogen DNA? It is likely that the two sources of DNA are differentially

located within the bone, the host within more heavily ossified intercellular structures than the pathogen. The partial decalcification step used in our extraction method might result in the discarding of the more superficial DNA in the sample. Empirical evidence leads us to reject this; a study using the method employed here extracted a set of nine femur samples which had been previously soaked for 5 min in a solution containing decreasing concentrations of the bacteriophage ΦX174 (Gilbert 2003). The extraction method was capable of detecting DNA at concentrations of an order equal to or less than 10^2 copies per millilitre of soaking liquid in eight samples. Further confidence in the extraction method is derived from the observation that sequences, presumably derived from environmental bacteria with an equally superficial distribution, are obtained after PCR.

An alternative possibility in the case of *M. tuberculosis* is that bacterial DNA is still encased in a lipid-rich cell wall, and this structure was not broken down by the enzymatic method employed in this study. This explanation is bolstered by the observation that many successful ancient *M. tuberculosis* papers employ a DNA extraction based on guanidium isothiocyanate/silica binding, a method posited to show an enhanced recovery of mycobacterial DNA from clinical samples. The principle flaw in this explanation is that it requires the cell wall to be completely preserved, an unlikely occurrence if the data on bacterial survival noted above (Duffield & Young 1985; Tanner & Michel 1999) are representative of the fate of resting *M. tuberculosis* in the environment, except under exceptional conditions such as recent, natural mummification (Donoghue *et al.* 2004). It should also be noted that many other studies successfully employ a wide variety of non-GuHCl methods to recover mycobacterial DNA (e.g. Kox *et al.* 1994; Hashimoto *et al.* 1995).

(7) While the majority of primer combinations used in this study have been taken directly from published studies dealing with ancient and modern extracts, it is possible that they are not sufficiently sensitive under the conditions employed here. However, the limits determined for these PCR assays suggest that they are highly sensitive, of an order equivalent to those previously published (table 2).

(8) The possibility that amplification of pathogen sequences was inhibited by components of the specimen not removed by DNA extraction can be rejected, as PCR amplifications were successful for both human DNA and untargeted microbial contaminant DNA. Further confirmation comes from the results of the amplification

	1	11	21	31	41	51	61	71
IB	TACAGCAATC	AACCCCTCAAC	TATCACACAT	CAACTGCAAC	TCCAAAGCCA	CCCCCTCACC	CACTAGGATA	CCAACAAACC
TB47 (n=5)						-		T.
TB53 (n=2)						-		
TB53 (n=4)						-		
TB57 (n=4)		TC.....	T.....			-		
TB57 (n=3)		C.....				-		
TB57 (n=3)						-		
TB58 (n=9)						-		
TB61 (n=2)		T.....				-..G..		
TB61 (n=6)						-		
TB82 (n=2)		T.....				-		
TB82 (n=2)						-		
TB82 (n=6)						-		
TB101 (n=10)		T.....				-		T.....
TB104 (n=2)		C.....				-	C.....	
TB104 (n=6)						-		
	81	91	101	111	121	131	141	
IB	TACCCACCCCT	TAACAGTACA	TAGTACATAA	AGCCATTTAC	CGTACATAGC	ACATTACAGT	CAAATCCC	
TB47 (n=5)	...T.....							
TB53 (n=2)	G.....		C.....					
TB53 (n=4)								
TB57 (n=4)			C.....					
TB57 (n=3)			C.....					
TB57 (n=3)								
TB58 (n=9)		G.....						
TB61 (n=2)	..T.....			..A.....				
TB61 (n=6)								
TB82 (n=2)								
TB82 (n=2)	T.....	C.....						
TB82 (n=6)								
TB101 (n=10)	T.....		G.....					
TB104 (n=2)			C.....					
TB104 (n=6)								

Figure 1. Sequence data for cloned PCR products from HTH extractions generated with the CR_16209/16356 primer pair. Relative to the topmost sequence (IB), 'dash' indicates a gap in the alignment at this position and 'dot' represents homology. See text for details.

of cervid DNA, as addition of the museum DNA templates did not in any case affect PCR success.

(a) *Implications of these data*

The absence of positive results from these analyses contrasts sharply with archaeological sample data, and particularly for TB, where high rates of detection are common (e.g. 55–75%: Haas *et al.* 2000; Zink *et al.* 2001a; Fletcher *et al.* 2003; Zink *et al.* 2003). These latter data compare favourably with published rates of detection using simple PCR-based systems on modern, diagnosed, clinical samples, which are around 80% (Portillo-Gomez *et al.* 2000; Van der Spoel van Dijk *et al.* 2000; Mitarai *et al.* 2001; Narayanan *et al.* 2001; Alfonso *et al.* 2002; Yee *et al.* 2002; Leung *et al.* 2003; Cheng *et al.* 2004), and higher than detection rates for blood (40%: Taci *et al.* 2003), urine (56%: Kafwabulula *et al.* 2002), and host DNA in studies of animal bones from temperate archaeological sites (around 10–20%: Haynes *et al.* 2002; Edwards *et al.* 2004). The high frequency of amplification success from archaeological samples has been attributed to the enhanced stability of the *M. tuberculosis* cell wall

(Donoghue *et al.* 2004). If so, further investigation needs to be directed at the specifics of the long-term preservation of the cell, and in particular its response to different environmental regimes.

Recovery of a number of non-target sequences, presumably derived from environmental contaminants, is also in contrast to previously published data. Previous reports of ancient pathogen DNA recovery mention sporadic problems with non-specific amplification, but these are relatively unusual (Mays *et al.* 2001). Most amplicons in these studies are clean enough to be directly sequenced without cloning. Data from the microbiology literature on the detection of mobile genetic elements suggest that co-amplification of multiple sequences is the most common result in investigations of environmental samples (Smalla *et al.* 2000). In this case, absolute identifications are made either by sequencing of multiple clones or by southern blotting; for ancient DNA, cloning and sequencing have to be considered the required standard for identifications while also providing additional information on template damage. Thus, it seems clear that determining the conditions by which specificity of

amplification is maintained should also constitute an area of investigation for the field.

It is not clear where the source of the disagreements between ours and previous studies lies, although we note that both an absence of positive amplifications, and the presence of non-specific amplicons have been reported in another study of ancient pathogens, an investigation of *Yersinia pestis* (Gilbert *et al.* 2004a). The authors of that study see their results as grounds for rejecting the claims of earlier work that identified *Y. pestis* from archaeological material (Drancourt *et al.* 1998; Raoult *et al.* 2000). We are more cautious in our conclusions, but would suggest that future work in this area concentrates on basic investigation of molecular taphonomy and explicit hypothesis testing. In addition to the questions noted above, experiments to establish that DNA damage does not alter strain profiles, particularly when spoligotyping (Kamerbeek *et al.* 1997), to verify that *M. bovis* is recovered from archaeological animal bones, and to establish that soil-dwelling *M. tuberculosis* is not a plausible contaminant of archaeological bone, should be undertaken.

5. CONCLUSION

This study describes markedly different results to those generally reported in studies of ancient pathogens, and suggests some ways, in which the causes of those observed differences might be identified. The next steps for the study of ancient bacterial pathogens are in both understanding the phenomena of microbial DNA survival over long time-scales, and in moving beyond diagnostic testing to actually use the data to examine evolutionary processes. It is unclear how easy this will be, as the utility of such studies in historically derived viral material is predicated on a high mutation rate. For bacteria, much lower rates of mutation have been estimated (Ochman *et al.* 1999), which hampers both the identification of contaminants, as noted above, and the application of many methods used in mapping strains and establishing population dynamics. Combined with the effects of rampant recombination in some taxa, the vagaries of the time-scale of divergence between bacterial species and the underlying demography of bacterial populations (Maiden *et al.* 1996; Falush *et al.* 2001), devising experiments that support the utility of palaeo-microbiology, must remain the major challenge and priority.

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