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The limits of biomolecular palaeopathology: ancient DNA cannot be used to study venereal syphilis

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Abstract

To determine whether ancient DNA (aDNA) can be used to study the palaeopathology of venereal syphilis, we carried out a comprehensive analysis of the preservation of human and pathogen DNA in a set of 46 bones of various ages, most of which displayed osteological indications of the disease. Bones came from seven English cemetery sites that were in use during the 9th–19th centuries. Twelve of the 46 bones consistently yielded mitochondrial DNA (mtDNA) sequences after replicate polymerase chain reactions (PCRs), and a further 13 bones yielded mtDNA sequences with less reproducibility. The sequence data enabled tentative mitochondrial haplogroups to be assigned to nine of the bones, and the identities and frequencies of these haplogroups were compatible with the geographical origins of the bones. Twenty-one bones consistently gave negative results with all mtDNA PCRs, indicating that at least these bones were not contaminated with modern human DNA, and those bones that gave positive results only yielded one sequence each, again suggesting that widespread modern contamination had not occurred. *Mycobacterium tuberculosis* sequences were obtained from seven bones, including three of five bones with tuberculous lesions. The cloned and direct sequences obtained from both the mtDNA and *M. tuberculosis* PCR products showed features typical of degraded aDNA. All of these results suggest that at least some of the 46 bones that we studied were suitable for aDNA analysis. All 46 bones were tested with nine different treponemal PCRs, each optimised to give a detection limit of ≤ 5 genomes. Although various bones gave PCR products of the expected size with one or more of these PCRs, sequencing showed that none of these products were authentic treponemal amplicons. Our failure to detect treponemal DNA in bones that were suitable for aDNA analysis, using highly sensitive PCRs, suggests that treponemal DNA is not preserved in human bone and that it is therefore not possible to use aDNA analysis to study venereal syphilis. Any past or future paper claiming detection of treponemal aDNA should therefore be accompanied by a detailed justification of the results.
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1. Introduction

Ancient DNA is looked on as having enormous potential in the study of palaeodisease [7]. Theoretically, any pathogen that invades the blood system and/or hard tissues could leave an ancient DNA signature in the skeletal remains after the death of the host. Detection of this ancient DNA could be used to identify the pathogen and hence assign a possible cause of death, and analysis of variable regions of the pathogen genome could, potentially, be used to study the evolution of disease-causing organisms.

To date, most of the attention in biomolecular palaeopathology has been focused on tuberculosis, with a growing body of literature describing use of the polymerase chain reaction (PCR) to amplify *Mycobacterium tuberculosis* complex DNA from human bones of

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various ages [2,6,14,16,17,19,25,37,38,42,44], but there have also been reports of detection of ancient DNA (aDNA) for leprosy [20,28,30,45], malaria [36,43], plague [15,31] and syphilis [22]. Because the aDNA being sought is pathogen rather than human, studies of palaeodisease are less susceptible to artefactual results due to contamination with modern DNA during handling of bones. However, such contamination is still possible, and the veracity of some published accounts of ancient pathogen DNA is difficult to assess as the comprehensive procedures required to authenticate the ancient origin of a DNA sequence [13] are not always described.

One of the most interesting palaeopathological questions that could be answered through use of aDNA is the origin of venereal syphilis in Europe. The first historical records for this disease are thought to relate to the epidemic of 1496, which affected large parts of Europe. As no equivalent disease of such virulence can be recognised earlier than 1496 it has been suggested that this epidemic represents the emergence of a new pathogen, *Treponema pallidum* subsp. *pallidum*, not previously present in Europe. The close proximity between the date of the epidemic and the return of the Columbus expedition in 1493 led to the popular view that venereal syphilis was introduced from the Americas by infected crewmen [1], but there is little solid support for this theory in the archaeological and historical records, and it is contradicted by those Old World skeletons, dated to pre-1493, that appear to show osteological signs of venereal syphilis [26,33]. Alternative theories are, therefore, that venereal syphilis was present in Europe before 1493 and that the epidemic occurred due to low population immunity or to a mutation that increased the virulence of *T. pallidum* subsp. *pallidum* [21,24], or that another treponemal disease which was present in Europe adapted to different climatic conditions and/or cultural practices to become venereal syphilis around the end of the 15th century [23]. These questions are potentially testable by aDNA analysis. The underlying hypothesis of the Columbian Theory — that *T. pallidum* subsp. *pallidum* was absent in the Old World prior to 1493 — can be tested by examining the pre-Columbian syphilitic skeletons for aDNA of this organism. If *T. pallidum* subsp. *pallidum* is present in the Old World pre-1493 then analysis of the aDNA sequences of those genes thought to underlie the virulence of the organism might reveal genetic changes responsible for the increase in venereal syphilis in the 15th and 16th centuries.

For aDNA analysis of palaeodisease to be successful, pathogens must be incorporated into bone material, either through remodelling during the latter stages of the disease (as probably occurs with tuberculosis and leprosy) or by transfer of a blood-borne pathogen to the bone matrix after death (a possible explanation of the reports of *Plasmodium* DNA in some skeletal remains). In the tertiary stages of treponemal diseases, when bone remodelling occurs, the number of organisms within the host is reduced and there is hence little opportunity for pathogen DNA to enter the bone [32]. Pathogen load is higher during the secondary stages of venereal syphilis but during this period there is very little remodelling and the only opportunity for treponemal DNA to enter bones would be via normal turnover. To determine whether these considerations place limitations on biomolecular studies of venereal syphilis, we carried out a comprehensive analysis of the preservation of human and pathogen DNA in 46 bones of various ages, most of which displayed osteological indications of venereal syphilis.

2. Materials and methods

2.1. Bones

Bones are listed in Table 1. All were taken from non-disarticulated skeletons. Those displaying treponemal pathology were obtained from six cemetery sites: Rivenhall, Kingston-upon-Hull, Magdalen Street, Farringdon Street, Newcastle Infirmary and Poultry One. Five tuberculous bones were also studied, from Farringdon Street, Poultry One and Royal Mint.

Rivenhall cemetery in Essex was in use during the 9th–19th centuries. The one recovered skeleton to show signs of treponemal disease (bone 204) has been radiocarbon dated to pre-1493 with 99% confidence [26]. Kingston-upon-Hull is a Friary site that was in use 1316–1539 and which yielded five treponemal skeletons, out of a total of 211 [5,27]. Magdalen Street cemetery, located in Norwich, dates from about 1100 to 1468 and contained approximately 1000 skeletons, of which six have treponemal pathology ([40]; A. Stirland, personal communication). From these three sites, most of the treponemal bones have non-specific lesions, but seven bones show more definite signs of venereal syphilis. These are Kingston-upon-Hull 1216, 932 and 1121 (lab references 52, 53, 66 and 67), all of which have signs of caries sicca, and Magdalen Street 68, 129, 227 and 305 (lab references 33, 35, 36 and 37), which show cranial lesions specific for venereal syphilis. However, only one of these bones, Kingston-upon-Hull 1216, has been radiocarbon dated to before 1493, and this date is disputed because of the marine carbon effect [3]. Most of the treponemal skeletons from Farringdon Street, London (1730–1849 [12]), Newcastle Infirmary (1753–1845 [29]) and Poultry One, London (12th century–1849, but mostly 18th–early 19th century skeletons [35]) have non-specific post-cranial lesions, but there is a high probability that these are due to venereal syphilis. Historical and medical records indicate that venereal syphilis was common in Britain in the 18th and 19th centuries, and non-venereal treponemes are unlikely to have been widespread in the indigenous population due

Table 1
Bones

Lab. reference	Site	Site reference	Skeletal element	Pathology
1	Poultry One	106	Tibia	Treponemal
2	Poultry One	160	Tibia	None
3	Poultry One	453	Humerus	Treponemal
4	Poultry One	601	Tibia	Treponemal
5	Poultry One	861	Tibia	Treponemal
9	Poultry One	519	Clavicle	None
10	Farringdon Street	1119	Tibia	Treponemal
11	Farringdon Street	1139	Tibia	Treponemal
12	Farringdon Street	1167	Tibia	Treponemal
13	Farringdon Street	1350	Ulna	None
17	Farringdon Street	1563	Humerus	Treponemal
18	Farringdon Street	1653	Clavicle	None
19	Farringdon Street	1653	Tooth	None
20	Farringdon Street	1817	Tibia	Treponemal
21	Farringdon Street	2298	Tooth	Treponemal
25	Newcastle Infirmary	68	Tibia	Treponemal
26	Newcastle Infirmary	129	Frontal	Treponemal
27	Newcastle Infirmary	157	Fibula	Treponemal
28	Newcastle Infirmary	200	Rib	None
29	Newcastle Infirmary	209	Rib	None
33	Magdalen Street	68	Femur	Treponemal
34	Magdalen Street	114	Rib	None
35	Magdalen Street	129	Fibula	Treponemal
36	Magdalen Street	227	Femur	Treponemal
37	Magdalen Street	305	Femur	Treponemal
41	Magdalen Street	349	Ulna	Treponemal
42	Magdalen Street	375	Rib	None
43	Magdalen Street	412	Fibula	Treponemal
44	Magdalen Street	434	Rib	None
45	Magdalen Street	496	Rib	None
49	Newcastle Infirmary	106	Tibia	Treponemal
50	Kingston-upon-Hull	805	?	None
51	Kingston-upon-Hull	913	?	None
52	Kingston-upon-Hull	1216	Left femur	Treponemal
53	Kingston-upon-Hull	1216	Right femur	Treponemal
57	Rivenhall	1389	Fibula	None
58	Rivenhall	204	Tibia	Treponemal
65	Kingston-upon-Hull	548	?	Treponemal
66	Kingston-upon-Hull	932	?	Treponemal
67	Kingston-upon-Hull	1121	?	Treponemal
68	Kingston-upon-Hull	1145	?	Treponemal
73	Royal Mint		Vertebra	Tuberculous
74	Poultry One	429	Vertebra	Tuberculous
75	Farringdon Street	1570	Vertebra	Tuberculous
76	Farringdon Street	1897	Rib	Tuberculous
77	Farringdon Street	2122	Rib	Tuberculous

to the temperate climate and cultural behaviour of this period.

Of the five tuberculous bones, *M. tuberculosis* DNA has previously been reported in the Royal Mint bone [42]. This site, the graveyard of the abbey of St Mary Graces, was founded in 1350 and closed in 1538.

2.2. DNA extractions

All samples were taken under aseptic conditions. The outer 1–2 mm of the bone was removed with a sterile scalpel and the bone then UV irradiated (254 nm, 120,000 μjoules cm^{-2}, 2×5 min with the bone rotated 180° between each exposure) to minimise the risk of contaminating DNA on the surface being carried over to the extraction process. Each bone was sealed in a DNA-free plastic bag and crushed into powder. Between 0.49–0.51 g of bone powder was measured into 1.5-ml microfuge tubes. Two additional empty tubes were used as blanks for each set of extractions.

DNA was extracted using a modification of the method of Yang et al. [47], which we have previously shown to be the most efficient of five tested methods for extraction of aDNA from this set of bones [4]. One millilitre of extraction buffer (0.5 M EDTA pH 8.0, 0.5% SDS, 100 μg ml^{-1} Proteinase K) was added to each bone sample and the contents mixed. After incubation with constant agitation at 55 °C for 24 h, each sample was centrifuged at 2000 r.p.m. for 5 min and 0.5 ml of the supernatant then transferred into a 15-ml Falcon tube containing 2.5 ml PB buffer (Qiagen). After mixing, 0.75 ml was added to a Qiaquick column (Qiagen) which was then centrifuged at 14,000 r.p.m. for 1 min and the buffer that had passed through the column discarded. The centrifugation was repeated until all the sample-buffer mix had passed through and no further buffer was collected. PE buffer (0.75 ml, Qiagen) was added to the column which was centrifuged as above and the run through discarded, with the centrifugation again repeated until no further wash buffer was collected. The column was then transferred to a new 1.5-ml microfuge tube and 50 μl EB buffer (10 mM Tris–HCl pH 8.5, Qiagen) added. After 1 min at room temperature the column was centrifuged at 14,000 r.p.m. for 1 min and the eluate collected. A further 50 μl of EB buffer was added and the incubation and centrifugation repeated. The two eluates were pooled giving a total volume of approximately 96 μl.

2.3. Polymerase chain reactions and analysis of PCR products

A series of PCRs was carried out with each bone extract to test for the presence of amplifiable human mitochondrial DNA (mtDNA), for *M. tuberculosis* DNA, and for *T. pallidum* subsp. *pallidum* DNA. Each PCR was individually optimised for magnesium ion content and annealing temperature, and the sensitivity of each *T. pallidum* PCR was determined with control DNA (Table 2). A standard PCR of 100 μl contained 5 μl of bone extract or 2 μl of modern control DNA (gift from S. Lukehart) or 2.5 μl of modern DNA extracted from human saliva samples, 1× buffer (75 mM Tris–HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20), the appropriate amount of $MgCl_2$, 200 μM each dNTP, 200 ng each primer, 1% bovine serum albumin and 2.5 units *Taq* DNA polymerase (MBI

Table 2
Details of PCRs

Target locus	Primers	Product length (bp)	Magnesium concentration (mM)	Annealing temperature (°C)	Sensitivity	Ref.
(a) Human mitochondrial DNA						
HVS1	5′-CCACCTGTAGTACATAAAAACCCA-3′	147	2.0	55	n.d.	[4]
	5′-GTGGGTAGGTTTGTTGGTATCCTA-3′					
(b) *Mycobacterium tuberculosis* complex DNA						
IS6110	5′-CCTGCGAGCGTAGGCGTCGG-3′	123	1.5	68	n.d.	[46]
	5′-CTCGTCCAGCGCCGCTTCGG-3′					
IS1081 external	5′-ACAGGCGAGCCCGGATCTGC-3′	141	2.0	58	n.d.	G.M. Taylor (pers. commun.)
	5′-TGGCGGTAGCCGTTGCGC-3′					
IS1081 internal	5′-CTGCTCTCGACGTTCATCGCCG-3′	113	1.5	58	n.d.	G.M. Taylor (pers. commun.)
	5′-TGGCGGTAGCCGTTGCGC-3′					
(c) *Treponema pallidum* subsp. *pallidum* DNA						
15 kDa lipoprotein gene	5′-GAGCAGGATGTCTCTATGAGTTATAAAGAG-3′	118	2.5	55	$>2-\leq 5$ genomes	[22]
	5′-GAAGCCACTACCGATGTGCGT-3′					
15 kDa lipoprotein gene	5′-TGAGCAGGATGTCTCTATGAGTT-3′	122	1.5	50	$>2-\leq 5$ genomes	This study
	5′-AAGAGAAGCCACTACCGATGT-3′					
*Tpr*J	5′-AACACCATCGCAAGCTGTGG-3′	142	1.5	55	≤ 2 genomes	This study
	5′-TGTACAGGTCATGGGTGAGG-3′					
*Tpr*J	5′-TGCACAGCTGCGTGCTGGT-3′	130	1.5	50	≤ 2 genomes	This study
	5′-GCCGATTCCCTGGGTGAGG-3′					
*Tpr*GJ	5′-TGCTAAACCTACCGGTAAGG-3′	157	2.0	58	$>2-\leq 5$ genomes	This study
	5′-GAAGGTGTTCATTACCGACC-3′	124				
	5′-TACCTGCTCAAACCCCTTCA-3′					
*Tpr*CDEFGIJ	5′-AACTTTGCCCAGCTGTGGAA-3′	189	2.0	58	≤ 2 genomes	This study
	5′-GTCAGTACTATCCCAGGCACC-3′					
*Tpr*CDFI	5′-CTTTGTCACCCGTGCCTATT-3′	109	2.0	60	≤ 2 genomes	This study
	5′-TACTCCGCTGTTTCCCACAT-3′					
*Tpr*EGJ	5′-AGGAGGATGCGGTACAGTAT-3′	117	1.5	56	≤ 2 genomes	This study
	5′-CAAAGGAGAGAAACCCCACA-3′					
*Tpr*K	5′-ATTAAAGTGGAGCTCACCGG-3′	112	2.5	58	$>2-\leq 5$ genomes	This study
	5′-GCTTCATACTCACCTTAGCC-3′					

For each primer pair, the upper primer is the forward primer and the lower is the reverse primer. The IS1081 internal primers are used to reamplify products obtained with the IS1081 external primer pair. For the *Tpr*GJ system, the upper primer is a forward primer specific for *Tpr*J of *T. pallidum* subsp. *pallidum*, and the middle primer is a forward primer that anneals to *Tpr*G of subsp. *pallidum* and *Tpr*J of subsp. *pertenue*. The lower primer anneals to *pallidum Tpr*G and *Tpr*J and *pertenue Tpr*J. The upper and lower primers give a 157 bp product and the middle and lower primers give a 124 bp product. The three primers can be used in a single PCR. The *Tpr*CDFI and *Tpr*EGJ primer pairs anneal to sequences within the region covered by *Tpr*CDEFGIJ and can be used to reamplify products obtained with the *Tpr*CDEFGIJ primers. HVS1, hypervariable segment 1; n.d., not determined.

Fermentas). For reamplifications, 2.5 μl of the initial PCR was included in a new 100 μl reaction. Cycling conditions were: 4 min at 94 °C; followed by 25 cycles (modern control PCRs and re-amplification PCRs) or 44 cycles (aDNA PCRs) of 1 min at the appropriate annealing temperature, 1 min at 72 °C and 1 min at 94 °C; followed by 1 min at the annealing temperature and 8 min at 72 °C. PCRs were examined by electrophoresis in 3% agarose gels. Bands were purified using Qiaquick columns (Qiagen), and DNA was ligated into pCR2.1 TOPO (Amersham) and cloned in *Escherichia coli* TOP10F′ cells. Recombinant plasmid DNA was purified using Qiaquick columns and sequenced with either the DYEnamic ET Terminator Cycle or ABI Big Dye sequencing kits (Amersham). Some PCR products were directly sequenced using these kits, without prior cloning. Sequences were aligned by eye.

2.4. Ancient DNA regime

Precautions were taken to minimise the risk of contaminating ancient material with modern DNA. Ancient extracts were prepared in an isolated room not used for handling modern DNA or PCR products. PCR mixes were set up in a third laboratory in a laminar flow cabinet (HEPA filter, Class 100, conforming to BS 5295 and 5726) used for no other purpose. Standard precautions were taken regarding pipette types. Solutions were sterilised by autoclaving, disposable plastics by UV irradiation, and work surfaces by washing in bleach. All ancient DNA amplifications were accompanied by an extraction and water blank. If any blank gave a PCR product then all accompanying test PCRs were discarded.

Table 3
Results of mtDNA PCRs

Bone	Reproducibility of results	Comparison with Cambridge Reference Sequence	Haplogroup
2	++	*16223*	I or W
4	+	16256, *16270*	U5
5	+	*16224*	K
9	+	None	?
10	++	None	?
12	+	None	?
13	+	None	?
17	++	None	?
20	++	16256, *16270*	U5
21	++	16189, *16224*	K
25	++	None	?
26	+	None	?
27	+	16256	?
28	++	None	?
29	+	None	?
33	++	16219	?
34	+	*16223*	I or W
37	+	None	?
43	+	*16223*	I or W
44	++	16171(deletion), *16176(G)*, *16223*	N1b
58	+	None	?
65	++	16193, 16219, 16265(T)	?
66	++	16263	?
76	+	*16189*, 16256	U1a or U2
77	++	None	?

Only the 25 bones that yielded mtDNA sequences are listed. For "Reproducibility of results", ++ indicates consistent results after replicate PCRs of replicate extracts, and + indicates that not all PCR products could be cloned though those that were gave consistent results. "Comparison with Cambridge Reference Sequence" lists the nucleotide positions at which the consensus sequence for each bone differs from the Cambridge Reference, using the standard numbering for human mtDNA. All changes from the Cambridge Reference were transitions except for those three for which the change is indicated in brackets after the nucleotide position. Changes in italic are indicative of the haplogroup(s) given.

3. Results and discussion

3.1. Presence of human mtDNA in the bones

There are approximately 8000 copies of the mitochondrial genome per human cell [8]. This is substantially higher than the anticipated relative copy number for treponemal genomes, so PCRs directed at a mitochondrial locus must be successful if there is to be any chance that treponemal aDNA will be detectable in a bone.

Each of the 46 bones listed in Table 1 was tested for the presence of human mtDNA by PCRs directed at the hypervariable segment 1 (HVS1). Evidence for the presence of mtDNA was obtained for 25 bones (Table 3). For 12 bones, replicate PCRs of replicate extractions always gave products of the correct size. The cloned products for each of these bones gave genuine HVS1 sequences, any differences between the sequences of clones from a single bone being attributable to misreading artefacts, due either to the basal error rate of DNA synthesis by the *Taq* DNA polymerase or to the presence in the template DNA of chemically-modified nucleotides resulting from DNA degradation. Another 13 bones gave less reproducible results, not all products being clonable but those that could be cloned giving consistent HVS1 DNA sequences.

Mitochondrial haplogroups can be assigned if the entire 340 bp HVS1 region is sequenced. The 147 bp segment amplified by the PCRs used in this study contains six of the polymorphic sites used for European and Near Eastern/West Eurasian haplogroup identification, and so enables tentative haplogroups to be assigned to some but not all of the sequences that are obtained. Nine of the sequences from these bones could be assigned to a single haplogroup or to two alternative haplogroups (Table 3), and all seven of these haplogroups were typical of West Eurasian populations.

The 25 positive bones gave a total of 11 different HVS1 sequences, taking into account all deviations from the Cambridge Reference Sequence. This is a similar pattern to that observed by us when this PCR system is used to study modern DNA samples [41], indicating that the sequence variability among these bones is typical of a random human population.

Prior to DNA extraction, the outer 1–2 mm of each bone was removed with a sterile scalpel and the bone then UV irradiated (see Section 2), a method that we have shown to be effective at removing modern contaminating DNA deposited on the surface of a bone (unpublished results). This does not ensure that any remaining DNA must be endogenous aDNA, but consideration of the results described above suggests that there is a high possibility that the mtDNA sequences that were obtained were indeed ancient. First, none of the sequences contained a T to C transition at position 16172 (according to the standard numbering for the human mitochondrial DNA sequence) that is present in the HVS1 of Abigail Bouwman, the only member of the research group to handle these bones. This source for the mtDNA sequences can therefore be discounted. The other possible route for contamination in the molecular biology lab is via airborne PCR products from previous amplifications. If, despite the physical isolation of these bones in a dedicated laboratory (see Section 2), such contamination has occurred, then we would expect the bones to display mixed HVS1 sequences, rather than each bone giving a single sequence. Similarly, if the sequences result from contamination during handling then we would expect at least a few bones to have mixed HVS1 sequences. Therefore, we conclude that the mtDNA PCRs give good evidence for the survival of aDNA in these bones.

3.2. Presence of *Mycobacterium tuberculosis* complex DNA

The most frequent successes in biomolecular palaeopathology have been with the detection of *M. tuberculosis* DNA in bones displaying lesions indicative of tuberculosis. These successes have been ascribed to the high pathogen load that is present when the bone changes occur and to the possibility that the mycobacterial capsule provides some protection against DNA degradation after death. In contrast, bones from individuals with treponemal infections would be expected to have a much lower pathogen content, and treponemes lack a capsule and so would not display preferential DNA survival after death. Treponemal aDNA should therefore be much more difficult to detect than that of *M. tuberculosis*. Five of the 46 bones included in this study had palaeopathological evidence for tuberculosis, and these were of similar ages and displayed similar degrees of preservation (based on macroscopic evidence) to several of the bones with indicators of venereal syphilis. If the age and preservation of the syphilitic bones is compatible with survival of treponemal aDNA then one would expect that mycobacterial DNA would also be detectable in the tuberculous bones.

Each of the 46 bones was tested for the presence of *M. tuberculosis* complex DNA with two PCR systems, both of which target multicopy insertion sequences and have previously been successfully employed to detect *M. tuberculosis* aDNA (Table 2). The first system amplifies a 123 bp product from IS6110 [46], and the second uses a semi-nested PCR to amplify products of 141 bp (1st round) and 113 bp (2nd round) from IS1081 (G.M. Taylor, personal communication).

Four bones, none of which had tuberculous lesions, gave PCR products of the expected size after the IS6110 PCR (Table 4). The cloned products from bone 21 yielded the expected IS6110 sequences (Fig. 1A), but two of the other three bones gave sequences that were not from *M. tuberculosis* and which did not correspond to any sequences in the DNA databases, and the remaining PCR product could not be cloned. Twenty-eight bones gave products after the 1st round of IS1081 amplification but only 11 of these gave results after the 2nd round of PCR. These PCR products were directly sequenced and seven (bones 21, 28, 66, 68, 75, 76 and 77) gave *M. tuberculosis* sequences (Fig. 1B). Three of these (75, 76 and 77) had tuberculous lesions. One of the other bones with lesions (73) gave a product after the 1st round of the IS1081 PCR but this product failed to reamplify in the 2nd round. The other tuberculous bone (74) gave negative results with all PCRs.

Although bone 73 showed clear osteological indications of tuberculosis, the DNA extract was prepared from a different part of the skeleton and hence the negative result with this sample is not surprising. For the other four tuberculous bones, samples were taken directly from the lesions. Additionally, the fact that the only bone to give positive results with both the IS6110 and IS1081 PCRs did not show indications of tuberculosis (bone 21) is far from unprecedented as previous papers have reported a relatively high frequency of detection of mycobacterial aDNA in skeletons without lesions [16]. The results shown in Table 4 are therefore not incompatible with the hypothesis that genuine mycobacterial aDNA is present in a subset of the bones. In addition, the cloned and direct sequences (Fig. 1) display deviations from the expected sequences, including unreadable positions in the direct sequences (indicated by Ns in Fig. 1B), typical features of degraded aDNA molecules that are present in low quantity. Therefore, we conclude that the *M. tuberculosis* PCRs give further evidence for the survival of aDNA in these bones.

Table 4
Results of *Mycobacterium tuberculosis* complex PCRs

Specimen	Tuberculous lesions	IS6110		IS1081		
		PCR products	Correct sequence	1st round products	2nd round products	Correct sequence
10	No	Yes	No	Yes	No	
11	No	Yes	No	No		
12	No	No		Yes	Yes	No
17	No	No		Yes	No	
18	No	No		Yes	No	
19	No	No		Yes	No	
20	No	No		Yes	No	
21	No	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
25	No	No		Yes	Yes	No
26	No	No		Yes	No	
28	No	No		**Yes**	**Yes**	**Yes**
33	No	No		Yes	No	
34	No	No		Yes	No	
35	No	No		Yes	No	
36	No	No		Yes	No	
37	No	No		Yes	No	
41	No	No		Yes	No	
42	No	No		Yes	No	
43	No	Yes	No	Yes	No	
44	No	No		Yes	No	
45	No	No		Yes	Yes	No
58	No	No		Yes	Yes	No
65	No	No		Yes	No	
66	No	No		**Yes**	**Yes**	**Yes**
68	No	No		**Yes**	**Yes**	**Yes**
73	Yes	No		Yes	No	
74	Yes	No		No		
75	Yes	No		**Yes**	**Yes**	**Yes**
76	Yes	No		**Yes**	**Yes**	**Yes**
77	Yes	No		**Yes**	**Yes**	**Yes**

Only those bones that either gave a positive result after one or more PCRs, and/or display tuberculous lesions are listed. Confirmed detections of *M. tuberculosis* complex DNA (i.e. PCR products of the correct sequence) are given in **bold**.

3.3. Absence of Treponema pallidum DNA

Each of the 46 bones was tested for the presence of *T. pallidum* DNA with nine different PCR systems (Table 2). Before use with the bone extracts, the optimal magnesium ion concentration and annealing temperature was determined for each system, so that detection sensitivities in the range of 2–5 genomes per PCR were attained. With such high sensitivities, positive results should be obtained if any treponemal aDNA is present in the bones.

Two PCRs targeted the 5′ flanking region of the 15 kDa lipoprotein gene, spanning a nucleotide difference that distinguishes *T. pallidum* subsp. *pallidum* from

(a) IS6110

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EXPECTED  CCTGCGAGCGTAGGCGTCGGTGACAAAGGCCACGTAGGCGAACCCTGCCCAGGTCGACACATAGGTGAGGTCTGCTACCCACAGCCGGTTAGGTGCTGGTGGTCCGAAGCGGCGCTGGACGAG
CLONE 1   .............................................-.............................................................................
CLONE 2   .............................................-.............................................................................
CLONE 3   .............................................-.............................................................................
CLONE 4   ......................................................T...............................C....................................
CLONE 5   ..................................N..........-.............................................................................
```

(b) IS1081

```
EXPECTED  CTGCTCTCGACGTTCATCGCCGCCTTGATGGGGGCTGAAGCCGA----CGCCCTGTGCGGGGCGGGCTACCGCGAACGCAGCGATGAGCGGTCCAATCAGCGCAACGGCTACCGCCA
21        ........................................A...----.N...................................................................
28        ............................................---A.N............................N......................................
66        ...................................N...N....----..................T........................G.........................
68        ..........-...-N...........-......AC........----..................T........................G.........................
75        .......N.................-.........C........----..................T.........T..N...........G.........................
76        .......N...........................C........----..................T........................G.........................
77        .......N...........................C........CCGA..................-.
```

Fig. 1. *M. tuberculosis* complex DNA sequences. (A) Sequences from the clones of the product obtained after IS6110 PCR of bone 21. (B) Direct sequences of products obtained after IS1081 PCRs of bones 21, 28, 66, 68, 75, 76 and 77. Dots indicate identities with the expected sequence and dashes are deletions. N indicates a position in a sequence electrophoretogram where two base peaks were of similar height preventing the correct nucleotide from being ascertained. The direct sequence for bone 77 is incomplete.

Table 5
Results of PCRs directed at treponemal DNA

Bone	Results of PCRs (product of expected size/confirmed as treponemal DNA)								
	15 kDa 1	15 kDa 2	*Tpr*J 1	*Tpr*J 2	*Tpr*GJ	*Tpr*CDEFGIJ	*Tpr*CDFI	*Tpr*EGJ	*Tpr*K
3	Yes/no		Yes/no						
4									Yes/no
10	Yes/no		Yes/no	Yes/no		Yes/no			Yes/no
11	Yes/no								
12	Yes/no		Yes/no			Yes/no			Yes/no
13			Yes/no		Yes/no	Yes/no			
17						Yes/no			
18									Yes/no
25						Yes/no			
26	Yes/no		Yes/no			Yes/no			Yes/no
27						Yes/no			
28	Yes/no				Yes/no	Yes/no			Yes/no
29						Yes/no			
33						Yes/no			
34					Yes/no	Yes/no			
35				Yes/no	Yes/no				
36			Yes/no	Yes/no					
37						Yes/no			Yes/no
41						Yes/no			
42					Yes/no				
44					Yes/no				
45				Yes/no					
49			Yes/no	Yes/no					
52			Yes/no	Yes/no		Yes/no			
53				Yes/no					
58		Yes/no							
65									Yes/no
67				Yes/no					Yes/no

other treponemes [9]. The other seven PCRs targeted one or more members of the *Tpr* gene family, which are potential virulence factors and hence of relevance to past changes in the pathogenicity of venereal syphilis. The *Tpr*J PCRs span nucleotide positions that distinguish subsp. *pallidum* from subsp. *pertenue*. The *Tpr*GJ PCR makes use of sequence similarities between the *Tpr*G and *Tpr*J genes in such a way that two products are generated from subsp. *pallidum* and one from subsp. *pertenue*. The *Tpr*CDEFGIJ, *Tpr*CDFI and *Tpr*EGJ PCRs use primers that anneal to a variety of *Tpr* genes, as indicated by the PCR designations, hence targeting multicopy positions in the *T. pallidum* genome and increasing the effective sensitivity of detection. The *Tpr*K PCR examines a member of the *Tpr* gene family that has attracted considerable interest as a determinant of virulence [10,11,39].

Seven PCRs (15 kDa 1, 15 kDa 2, *Tpr*J 1, *Tpr*J 2, *Tpr*GJ, *Tpr*CDEFGIJ and *Tpr*K) gave products of the expected size with some bones, but none of these products could be confirmed as true treponemal DNA amplicons. Attempted confirmation was by sequencing of cloned PCR products for all except *Tpr*CDEFGIJ, for which confirmation was attempted by reamplification of products with the *Tpr*CDFI and *Tpr*EGJ primer pairs, which target sequences internal to *Tpr*CDEFGIJ. The *Tpr*CDFI and *Tpr*EGJ PCRs gave no products of the expected size. These results are summarised in Table 5. Negative results have also been obtained with bones 50–53, 57, 58 and 65–68, and two additional bones from Ipswich Blackfriars, in a separate project by Dr G.M. Taylor (Imperial College School of Medicine, London), using primers directed at the region of the treponemal *tpp* gene whose amplication from bone material was reported by Kolman et al. [22] (G.M. Taylor, personal communication).

In view of the abilities of the PCRs that we used to detect as little as 2–5 genomes of authentic modern treponemal DNA, our failure to obtain an authentic treponemal PCR product in any experiment must be taken as strong evidence that treponemal DNA was not present in any of the bones that we studied.

4. Conclusions

The aim of the work described in this paper was to determine if ancient DNA can be used to study the palaeopathology of venereal syphilis. We assembled a set of relatively recent bones, many with osteological indications of venereal syphilis, and tested these for the presence of human mtDNA, *M. tuberculosis* DNA and treponemal DNA. The results are summarised in Table 6. We carried out nine different treponemal PCRs

Table 6
Summary of PCR results

Bone	Pathology	Authentic PCR results		
		MtDNA	*M. tuberculosis* DNA	Treponemal DNA
1	Treponemal	No	No	No
2	None	Yes	No	No
3	Treponemal	No	No	No
4	Treponemal	Possibly	No	No
5	Treponemal	Possibly	No	No
9	None	Possibly	No	No
10	Treponemal	Yes	No	No
11	Treponemal	No	No	No
12	Treponemal	Possibly	No	No
13	None	Possibly	No	No
17	Treponemal	Yes	No	No
18	None	No	No	No
19	None	No	No	No
20	Treponemal	Yes	No	No
21	Treponemal	Yes	Yes	No
25	Treponemal	Yes	No	No
26	Treponemal	Possibly	No	No
27	Treponemal	Possibly	No	No
28	None	Yes	Yes	No
29	None	Possibly	No	No
33	Treponemal	Yes	No	No
34	None	Possibly	No	No
35	Treponemal	No	No	No
36	Treponemal	No	No	No
37	Treponemal	Possibly	No	No
41	Treponemal	No	No	No
42	None	No	No	No
43	Treponemal	Possibly	No	No
44	None	Yes	No	No
45	None	No	No	No
49	Treponemal	No	No	No
50	None	No	No	No
51	None	No	No	No
52	Treponemal	No	No	No
53	Treponemal	No	No	No
57	None	No	No	No
58	Treponemal	Possibly	No	No
65	Treponemal	Yes	No	No
66	Treponemal	Yes	Yes	No
67	Treponemal	No	No	No
68	Treponemal	No	Yes	No
73	Tuberculous	No	No	No
74	Tuberculous	No	No	No
75	Tuberculous	No	Yes	No
76	Tuberculous	Possibly	Yes	No
77	Tuberculous	Yes	Yes	No

on each of 46 bones but none of these gave an authentic positive result. There are three possible explanations for the failure to detect treponemal DNA:

1. *The bones were not suitable for aDNA analysis, because they do not contain aDNA or because they contain inhibitors that prevent amplification of aDNA.* This explanation appears to be unlikely in view of the results that we obtained with PCRs directed at human mtDNA and *M. tuberculosis* DNA. Twelve of the 46 bones consistently yielded mtDNA sequences after replicate PCRs directed at the mitochondrial HVS1, and a further 13 bones yielded sequences after some PCRs. The sequence data enabled haplogroups to be assigned to nine of the bones, and the identities and frequencies of these haplogroups were compatible with the geographical origins of the bones. Twenty-one bones gave negative results with all mtDNA PCRs, but only one of these bones (57) contained inhibitors of PCR, as shown by experiments in which bone extracts were added to control PCRs with modern DNA. These results indicate that at least 20 of the bones were not contaminated with modern human DNA. Those bones that gave positive results only yielded one HVS1 each, again suggesting that widespread modern contamination (which would be expected to result in mixed sequences) had not occurred. *M. tuberculosis* sequences were obtained from seven bones, including three of the five bones with tuberculous lesions. The cloned and direct sequences obtained from both the mtDNA and *M. tuberculosis* PCR products showed features typical of degraded aDNA. All of these results suggest that at least some of the 46 bones that we studied were suitable for aDNA analysis.
2. *Treponemal aDNA was present but the PCR systems were insufficiently sensitive to detect this DNA.* This explanation appears to be unlikely in view of the extreme sensitivities of the nine treponemal PCRs that we used, as shown by control experiments with modern DNA. PCRs were optimised to give detection limits of 2–5 genomes or less, and three of the PCRs targeted multicopy sequences in the treponemal genome, meaning that, in effect, their detection limits were less than one genome. It is difficult to imagine how a more sensitive set of PCRs could be developed for detection of treponemal DNA.
3. *There was no treponemal DNA in the bones.* This appears to be the unavoidable conclusion from our experiments. Each PCR was carried out with the equivalent of 12.5 mg of bone material, hence even for the least sensitive PCRs a negative result indicates a treponemal DNA content of <1 genome per 2.5 mg bone. One could construct a pedantic argument about the definition of "no DNA", but any reasonable assessment of our results has to conclude that treponemal aDNA simply was not present in these bones.

Many positive detections of pathogen aDNA have been reported in the literature (see Section 1). Some of these reports are undoubtedly correct, but others lack a description of the rigorous validation experiments that are recommended for aDNA research (e.g. [13]), raising questions about the authenticity of the detections that

are described. Few papers that report negative results in the search for pathogen aDNA (or indeed for any type of aDNA) have been published. The only equivalent study that we are aware of is that of Gilbert et al. [18], who failed to identify *Yersinia pestis* DNA in teeth from supposed plague victims. Negative results are nonetheless important as they may indicate the limits of biomolecular palaeopathology and hence enable the authenticity of papers reporting unsupported detections to be assessed. The outcomes of the experiments described in our paper suggest that treponemal DNA is not preserved in human bone and that it is therefore not possible to use aDNA analysis to study venereal syphilis. The specific conclusion is that any past or future paper claiming detection of treponemal aDNA should be accompanied by a detailed justification of the results. A more general conclusion is that, as we have previously suggested [34], it is easy to obtain deceptive results when using PCR to study palaeodisease, and if biomolecular palaeopathology is to become a fully mature, fully trusted research discipline, then all projects must incorporate rigorous planning and careful interpretation of results.

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