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Absence of *Yersinia pestis*-specific DNA in human teeth from five European excavations of putative plague victims

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This study reports the results of a collaborative study undertaken by two independent research groups to (a) confirm recent PCR-based detection of *Yersinia pestis* DNA in human teeth from medieval plague victims in France, and (b) to extend these observations over five different European burial sites believed to contain plague victims dating from the late 13th to 17th centuries. Several different sets of primers were used, including those previously documented to yield positive results on ancient DNA extracts. No *Y. pestis* DNA could be amplified from DNA extracted from 108 teeth belonging to 61 individuals, despite the amplification of numerous other bacterial DNA sequences. Several methods of extracting dentine prior to the DNA extraction were also compared. PCR for bacterial 16S rDNA indicated the presence of multiple bacterial species in 23 out of 27 teeth DNA extracts where dentine was extracted using previously described methods. In comparison, positive results were obtained from only five out of 44 teeth DNA extracts for which a novel contamination-minimizing embedding technique was used. Therefore, high levels of environmental bacterial DNA are present in DNA extracts where previously described methods of tooth manipulation are used. To conclude, the absence of *Y. pestis*-specific DNA in an exhaustive search using specimens from multiple putative European plague burial sites does not allow us to confirm the identification of *Y. pestis* as the aetiological agent of the Black Death and subsequent plagues. In addition, the utility of the published tooth-based ancient DNA technique used to diagnose fatal bacteraemias in historical epidemics still awaits independent corroboration.

INTRODUCTION

The Black Death is the name given to a pandemic which killed up to a third of the European population between 1347 and 1352 (Cohn, 2002; Scott & Duncan, 2001). Over the next three hundred years this pandemic was followed by further plagues (deadly epidemics or pestilences) of lesser mortality. Whether these historically described diseases correspond to bubonic plague, whose aetiological agent was identified to be the bacterium now known as *Yersinia pestis* by Alexandre Yersin in 1894 (Yersin, 1894), has been the subject of controversy (Cohn, 2002; Scott & Duncan, 2001). Recently, DNA specific for *Y. pestis* was amplified from 16th and 18th century human teeth believed to be from French plague victims (Drancourt *et al.*, 1998) and 14th century French Black Death victims (Raoult *et al.*, 2000). The lead authors of these reports believe that the consideration of any cause for the Black Death other than *Y. pestis* is now speculative (Raoult & Drancourt, 2002).

The aetiology of the Black Death is of major historical interest, but there are other significant consequences of resolving the issue of attribution. Knowledge of aetiology is

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Abbreviations: ABC, Ancient Biomolecules Centre; aDNA, ancient DNA; SBH, St Bartholomew's Hospital.

Details of all cloned bacterial DNA sequences and database matches and specific details of PCR cycles are available in *Microbiology* Online.

important to understand any possible evolutionary impact of the associated mortality (Stephens *et al.*, 1998) and to assess our ability to control any similar contemporary disease. Many scientific resources are now available to fight a future re-emerging human pandemic of *Y. pestis* infection. Well-established protocols exist to diagnose, treat and prevent transmission (WHO, 1999), the genome has been sequenced (Parkhill *et al.*, 2001; Deng *et al.*, 2002) and a subunit vaccine is in clinical trials (Titball & Williamson, 2001). These resources would be ineffective if a re-emerged Black Death had a different aetiology. Therefore, for historical and public health reasons alone, independent replication of the observations of *Y. pestis* DNA in ancient remains from a small geographical area seems desirable. The nature of ancient DNA (aDNA) evidence makes independent confirmation a necessity.

The study of aDNA involves extraction and analysis of DNA from the remains of organisms preserved as fossils, skeletons or mummified tissues. Pathogen DNA has been reported in a range of ancient animal and human remains. Many of the reports are from skeletons exhibiting paleopathological evidence of the disease. This includes reports of *Mycobacterium leprae* DNA from skeletal remains (Rafi *et al.*, 1994) and DNA from various members of the *Mycobacterium tuberculosis* complex from human skeletal (Salo *et al.*, 1994; Taylor *et al.*, 1996), bison skeletal (Rothschild *et al.*, 2001) and human mummified tissue (Nerlich *et al.*, 1997; Fletcher *et al.*, 2003a, b). In most of these cases structurally un-modified bone also yielded mycobacterial DNA. In the case of *Y. pestis*, fatal infection would not be expected to leave any specific bony changes, so no osteological confirmation is available and any retrospective diagnosis is completely DNA-based.

aDNA studies are hampered by extremely low levels of DNA preservation, often coupled with the presence of much greater levels of modern contaminants. Characteristically, only short aDNA fragments (less than 300 bp) can be amplified (Richards *et al.*, 1995; Höss *et al.*, 1996; Hofreiter *et al.*, 2001) and easy amplification of longer fragments is an indication that contamination has occurred. Contaminants normally arise from three sources; (i) modern equivalents of the source species, (ii) previously PCR-amplified DNA (amplicons) from the source species, or (iii) similar species in the environment (of especial importance in the study of micro-organisms) (Gutierrez & Marin, 1998). Unrecognized contamination as a source for positive results is so insidious and difficult to prevent that in many cases it only comes to light when results from one laboratory cannot be confirmed by other groups and a laboratory-specific contaminant is revealed (Austin *et al.*, 1997; Gutierrez & Marin, 1998). A series of strict criteria have been proposed for research standards in this field (Cooper & Poinar, 2000). Possibly the most important of these criteria is the independent replication of results by other groups.

Two studies from the same research group have been published reporting the successful extraction, amplification and direct sequencing from PCR products of *Y. pestis*-specific DNA retrieved from the dental pulp cavities of plague victims (Drancourt *et al.*, 1998; Raoult *et al.*, 2000). Findings that pathogen-specific DNA can be recovered from this source in systemically infected animals (Aboudharam *et al.*, 2000) have led Drancourt *et al.* (1998) to hypothesize that teeth provide a lasting, contamination-free refuge where pathogen aDNA may survive.

This study presents the results of attempts by two independent research groups to amplify and sequence ancient *Y. pestis* DNA from human teeth. Samples were obtained from five archaeological sites in Northern Europe and were analysed using a range of PCR primers that were designed to be *Y. pestis*-specific. Three methods of aDNA extraction are also both directly and indirectly compared in order to assess the suitability of each method in preventing contamination of the DNA source.

METHODS

Samples. Teeth were chosen as sources of aDNA as in previous studies. Most teeth used were erupted, i.e. teeth from adults. In the first publication from the Marseille group, unerupted teeth, i.e. mainly teeth from non-adults, were used because these were thought to be better protected from environmental contamination (Drancourt *et al.*, 1998), but the second Marseille publication reported satisfactory results with adult erupted teeth from 14th century remains (Raoult *et al.*, 2000) and we attempted replication on this type of specimen. A range of samples from five archaeological sites were analysed to help ensure that at least some of the individuals died as a result of a plague infection (Table 1). The majority of samples (77/108) originated in two well-documented plague burial sites in Copenhagen and London. Four human teeth samples from a Viking burial site in York, which antedate the arrival of the Black Death in Britain (pre-11th century), and two human teeth from Farringdon (London) buried long after the last local plague outbreak (19th century) were also analysed as negative controls. Samples from these archaeological sites are referred to as 'YO' and 'FA', respectively, in this study.

Copenhagen site. Building construction in Copenhagen, Denmark, led to the excavation of a cemetery in 1991. Archaeological identification of this cemetery as the historically-recorded Vodroffsgaard plague cemetery was possible due to the geographical location, historical records and burial finds such as contemporary coffins (Ringboel Bitsch, 1991). This cemetery dates from the last major plague epidemic in Denmark, which struck Copenhagen in 1711–1712 AD. At that time, Vodroffsgaard was a farmstead outside the city walls, and the farm and property were requisitioned by the authorities to be used as a plague hospital and cemetery (Von Kohl, 1911). The excavations in 1991 revealed 54 graves. Subsequent anthropological analyses showed that the extracted human remains represented 57 individuals [13 children and adolescents, 20 adult males and 24 adult females (Lynnerup, 1992)]. Copenhagen samples are referred to as 'CP' in this study.

Royal Mint site. Purchase and consecration of land for a burial ground at East Smithfield (to the east of the walled city of London) is recorded during the first Black Death epidemic of 1349 (Hawkins, 1990). The East Smithfield cemetery on the site of the old Royal Mint was excavated between June 1986 and June 1988 revealing a mass burial pit, two mass burial trenches and 14 rows of

Table 1. Summary of samples examined in this study

Site*	Country†	Abbreviation‡	Details§	Individuals‖	Teeth¶	Source#
Copenhagen	Denmark	CP	Plague pit	34	50	N. Lynnerup
Royal Mint, London	England	RM	Plague pit	8	27	W. White
Verdun	France	VE	Plague pit	13	14	E. Carniel, F. Adam
Angers	France	AN	Plague pit/catastrophe grave	1	5	H. Mollaret
Spitalfields, London	England	SP	Plague pit/catastrophe grave	5	12	W. White
York	England	YO	Negative control	2	4	A. Cooper
Farringdon, London	England	FA	Negative control	1	2	W. White

*Archaeological site of origin of specimens.
†Country of origin of specimens.
‡CP, Copenhagen; RM, Royal Mint; VE, Verdun; AN, Angers; SP, Spitalfields; YO, York; FA, Farringdon.
§Specific details of site.
‖Number of individual skeletons from which teeth were extracted.
¶Total number of teeth investigated for DNA.
#Original source of samples.

stratigraphically contemporary individual graves. A total of 600 individuals were excavated and these were the source for our specimens. Previous studies on these remains have provided much useful archaeological and anthropological data on Black Death plague victims (Hawkins, 1990; Waldron, 2001). Royal Mint samples are referred to as 'RM' in this study.

Verdun site. Excavations in Verdun, north-eastern France, of the Hospice Sainte Catherine on a monastery site revealed several multiple graves and two burial pits containing 21 and 26 individuals, respectively. Burial pits of this kind on ancient hospital sites are associated with fatal disease epidemics. The teeth processed were from the larger burial pit, dated from clothing remnants and other artefacts to be from the late 17th/early 18th century. Monastery archives document 16 outbreaks of plague on the site from the 16th to the 18th century, making this a probable plague pit. Verdun samples are referred to as 'VE' in this study.

Angers site. Minor earthworks in the Place de la Paix, Angers, France, in 2001 revealed a mass grave of up to 1000 individuals that was not recorded in the city archives. Although archaeological analysis has not yet been undertaken, the large scale and unrecorded nature of the burial site represents a catastrophic event in a city noted for multiple severe plague outbreaks (Scott & Duncan, 2001), making this a possible plague pit. Angers samples are referred to as 'AN' in this study.

Spitalfields site. The Spitalfields site is located just outside the walls of Roman and medieval London. The main medieval findings relate to the priory and hospital of St Mary Spital founded in 1197 and dissolved by Henry VIII in 1538. Excavation by the Museum of London 1999–2001 revealed a large burial ground adjacent to the hospital that appears to have been in use throughout this period. One region of the burial ground is a mass grave with multiple skeletons buried together in a disorderly fashion suggesting mortality in a major epidemic. Carbon-14 dating of remains from this part of the excavation assigns them to the late 13th century (i.e. slightly earlier than 1349 when the Black Death was first recognized in London). Spitalfields samples are referred to as 'SP' in this study.

DNA extraction and analysis. Samples were analysed independently by two groups to control for laboratory-specific failures, Oxford University's Ancient Biomolecules Centre (ABC) and St Bartholomew's Hospital (SBH), London. The ABC is a dedicated aDNA facility where no work on modern DNA is ever undertaken. At SBH, most aDNA extraction and PCR set-up was undertaken in a specialized virology laboratory where no bacterial work is undertaken. Strict aDNA protocols were followed by both groups (Cooper & Poinar, 2000). This included the independent analysis of pairs of teeth from eight individuals excavated at three archaeological sites by both groups.

Assessment of dentine extraction techniques to minimize DNA extract contamination. Teeth samples must be powdered prior to DNA extraction. To remove surface contamination teeth were treated with bleach – those used at the ABC through immersion for 10 min in 50 % bleach solution, those at SBH were wiped but not immersed in 50 % bleach. Post-cleansing all teeth were exposed to UV light at λ 325 nm for 20 min. Three methods of tooth dentine extraction were then performed at the ABC to compare efficiency at (a) preventing contamination entering the DNA extract and (b) retrieving DNA.

(i) Eighteen teeth (14VE/2YO/2CP) were directly ground into powder ('ground') using a microdismembrator (Braun).

(ii) Nine teeth (5VE/2YO/2CP) were powdered using the method pioneered by Drancourt *et al.* (1998). This consists of pulp cavity removal by scraping with a dental tool following longitudinal fracturing of teeth ('scraped').

(iii) Fifty-three teeth (5AN/2RM/2SP/44CP) were encased in silicone rubber ('silicone') prior to the removal of dentine with a dental drill as reported in Gilbert *et al.* (2003b) (Fig. 1). This method of powder extraction was developed due to concerns about the efficiency of contaminant removal from the porous outer surface teeth and that surviving contaminant DNA may be transferred to extractions as the teeth are handled.

The efficiency of the methods was compared and contrasted by performing extractions using more than one method on some teeth and testing multiple teeth extracted from single skulls to ensure that the results were not sample- or site-specific. A total of 69 different teeth from possible plague sites were therefore processed at the ABC.

To directly compare extracts from 'scraped' and 'ground' teeth, seven teeth from seven individuals were first 'scraped', following which the remains were 'ground'. To compare all three methods, two teeth were

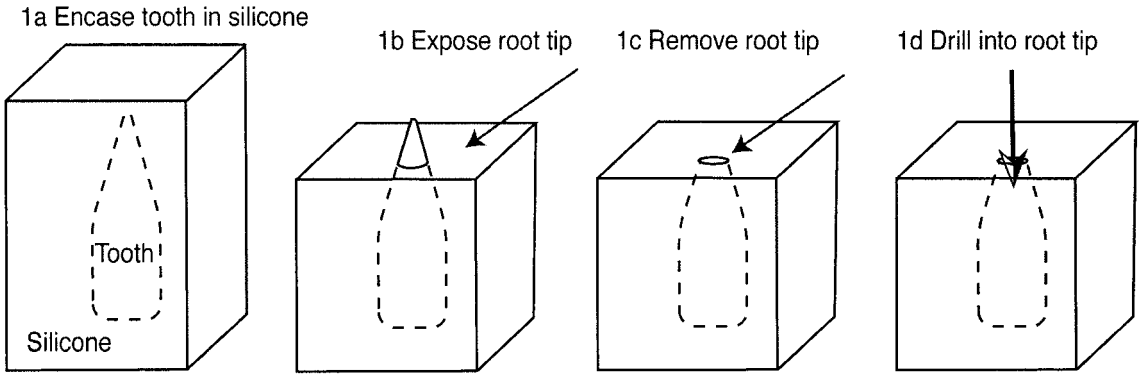

Fig. 1. Silicone extraction method used to remove dental pulp residue and dentine from teeth samples. Reproduced with permission from Gilbert *et al.* (2003b).

taken from each of two individuals. The first tooth from each individual was 'scraped' to remove some dentine, after which the remainder of the tooth was 'ground' (as above). Dentine was removed from the second tooth using the 'silicone' method. As a further control, two of the samples that were 'scraped' then 'ground' were from the burial site at York that is not believed to have contained victims of the plague.

DNA extractions at the ABC. DNA extractions on the powdered samples followed a standard phenol/chloroform method modified for aDNA samples, as described by Cooper *et al.* (2001).

Dentine removal at SBH. In total 39 medieval teeth were examined at SBH. Teeth were wiped with bleach and then exposed to UV light as at the ABC. The 'scrape' method used in the initial *Y. pestis* aDNA study (Drancourt *et al.*, 1998) was used to obtain dentine for DNA extraction from 17 medieval specimens and two 19th century Farringdon control teeth (DNA extractions BT1–BT24). Subsequently the ABC 'silicone' encasement technique was adopted for 22 further teeth (DNA extractions BT25–BT46).

DNA extractions at SBH. DNA extractions BT1–BT10 employed the technique described in the study by Drancourt *et al.* (1998). DNA extractions BT12–BT24 employed a different method, whereby dentine was placed into 1·5 ml DNA extraction buffer (5 M guanidinium isocyanate, 1·3 % Triton X-100, 0·1 M Tris/HCl pH 6·4, 0·02 M EDTA pH 8·2) and agitated at 48 °C overnight. Subsequently DNA recovery followed a silica-based extraction protocol (Boom *et al.*, 1990) modified for aDNA (Höss *et al.*, 1996). An intermediate pre-amplification step after DNA extraction and before PCR using random 15-mers following the method of Zhang *et al.* (1992) was also applied to these specimens only (BT12–BT24) and not to any other specimens processed. The remaining DNA extractions (BT25–BT46) were undertaken using a commercial kit (GENECLEAN Kit for Ancient DNA; Qbiogene).

PCR amplification

Primers. The presence of *Y. pestis* DNA in DNA extracts was monitored using PCR amplification, followed by cloning and sequencing where relevant. Primers, taken from both published aDNA studies, and of new design, were employed against three bacterial targets. One of these was not specific for *Y. pestis* – targeting part of the 16S rRNA gene. The other two were *Y. pestis*-specific: the plasmid-encoded plasminogen activator gene (*pla*) and the chromosomal RNA polymerase β-subunit-encoding gene (*rpoB*). All primer sets were optimized on a small sample of extractions. Samples were also assayed for endogenous human DNA using a selection of human mitochondrial Hyper Variable Region 1 (HVR-1) and nuclear β-globin DNA primers. For full primer details refer to Table 2. Specific details of PCR cycles are available in *Microbiology* Online (http://mic.sgmjournals.org).

Positive controls. The use of positive controls was avoided at the ABC in order to reduce the problem of sample cross-contamination that has been reported in aDNA studies (Kolman, 1999). However, two positive controls were initially used at SBH (in a separate building to that used for DNA extraction) for optimizing PCRs. One control was designed to be used with published primers (Drancourt *et al.*, 1998) (PlaB control), the other was designed for this study (PlaA control). Both consisted of target DNA, though with a central deletion removing a *Dra*I site and inserting a *Hin*dIII site, thus yielding smaller amplicons than the target.

PCR amplification, cloning and sequence analysis – ABC. The amplification, re-amplification, cloning and PCR methodology used at the ABC are as reported elsewhere (Cooper *et al.*, 2001). Where PCR products yielded multiple DNA bands, those of the expected size were excised from the gel for purification and re-amplification. All PCR products of the expected size were sequenced. Twenty-three PCR products from the ABC were also cloned prior to sequencing. Attempts were made to identify all sequences using the NCBI BLAST tool.

PCR amplification, cloning and sequence analysis – SBH. At SBH initial PCR (DNA extracts BT1–BT10) was performed with Ampli*Taq* (Applied Biosystems), later switching to Ampli*Taq* Gold (Applied Biosystems) (DNA extracts BT12–BT24) and latterly Platinum *Taq* Hifidelity (Invitrogen) (DNA extracts BT25–BT46) as at the ABC. In all cases, PCR was performed in 25 μl volumes with 1 μl DNA extract. For DNA extracts BT1–BT24, the PCR mix comprised 2·5 units of *Taq* in Perkin Elmer PCR buffer with 1·5 mM $MgCl_2$, 200 μM of each dNTP and 2 μM of each oligonucleotide primer.

RESULTS

Suitability of dentine extraction techniques

The amplification results from seven teeth where dentine was both 'scraped' and 'ground' prior to DNA extraction

Table 2. Primers used in this study

1, Research group where primer pair was used; 2, designated primer name; 3, primer pair abbreviation code; 4, annealing temperature of primers; 5, predicted amplicon (bp); 6, 5′→3′ sequence of primer; 7, use of positive control at any time in study with this primer set. Further PCR cycle parameters are available in *Microbiology* Online.

Group[1]	Primer[2]	Code[3]	°C[4]	Amp[5]	Sequence[6]	+ve control[7]	Source
ABC	Yprpob1*	A	58	134	aac acc tta tcg tcg tgt acg t	N	Drancourt *et al.* (1998)
ABC	Yprpob2*	A	58	134	aat ctt cta aaa agc ggc ctt ca	N	Drancourt *et al.* (1998)
ABC	Yprpob267*	B	58	140	gga aac acc tta tcg tcg tgt acg	N	This study
ABC	Yprpob403*	B	58	140	cca aat ctt cta aaa agc ggc c	N	This study
ABC	Yp16S387H†	C	58	214	ccg cgc tta acg tgg gaa c	N	This study
ABC	Yp16S604L†	C	58	214	gtc gac atc gtt tac agc	N	This study
ABC	Yp16S586H†	D	58	195	gct gta aac gat gtc gac	N	This study
ABC	Yp16S786L†	D	58	195	ctg tgg atg tca aga gta gg	N	This study
ABC/SBH	YPpla728H (YP12D)‡	E	52	148	cag cag gat atc agg aaa ca	N	Raoult *et al.* (2000)
ABC/SBH	YPpla876L (YP11R)‡	E	52	148	gca ag tcc aat ata tgg cat ag	N	Raoult *et al.* (2000)
ABC	L16131§	F	56	126	cac cat gaa tat gta cgg t	N	Handt *et al.* (1996)
ABC	H16218§	F	56	126	tgt gtg ata gtt gag ggt tg	N	Handt *et al.* (1996)
ABC/SBH	L16209§	G	56	184	cca tgc tta caa gca ag	N	Handt *et al.* (1996)
ABC/SBH	H16356§	G	56	184	gtc atc cat ggg gac gag aa	N	Handt *et al.* (1996)
SBH	Pla A (K1)‡	H	59	178	aag gag tgc ggg taa tag gt	Y	This study
SBH	Pla A (K2)‡	H	59	178	gat gtc ttc tca cgg aaa gt	Y	This study
SBH	Pla B (Pla1)‖	I	53	300	ctt gga tgt tga gct tcc ta	Y	Hinnebusch & Schwan (1993)
SBH	Pla B (Pla2)‡	I	53	300	gag atg ctg ccg gta ttt cc	Y	Drancourt *et al.* (1998)
SBH	Pla C (7242F)(YP11D)‡	J	52	148	cta tgc cat ata ttg gac ttg c	N	Raoult *et al.* (2000)
SBH	Pla C (7389R)(YP10R)‡	J	52	148	gag ccg gat gtc ttc tca cg	N	Raoult *et al.* (2000)
SBH	PCO3¶	K	56	110	aca caa ctg tgt tca cta gc	Y	Greer *et al.* (1991)
SBH	PCO4¶	K	56	110	caa ctt cat cca cgt tca cc	Y	Greer *et al.* (1991)
SBH	blaTEM 1-20#	L	55	858	atg agt att caa cat ttc cg	N	Essack *et al.* (2001)
SBH	blaTEM 858-840#	L	55	858	cca atg ctt aat cag tga c	N	Essack *et al.* (2001)

*Primer for *Y. pestis* RNA polymerase gene.
†Primer for *Y. pestis* 16S rRNA gene.
‡Primer for *Y. pestis* plasminogen-activator-encoding gene (*pla*).
§Primer for *Homo sapiens* mitochondrial HVR-1 region.
‖Primer for *Y. pestis* transcriptional regulator adjacent to *pla*.
¶Primer for *H. sapiens* nuclear β-globin.
#Primer for bacterial plasmid RP4.

highlight that neither method reduces the presence of amplifiable contaminant DNA (Table 3). However, the direct comparison between DNA extracts from samples where dentine was extracted from 'scraped' and 'silicone' teeth shows a clear reduction in levels of contaminants in 'silicone' treated teeth (Table 3). Following this observation, all further ABC and SBH work utilized the 'silicone' method.

Y. pestis PCR results

In total, DNA extractions were performed on 18 'ground' (14VE/2YO/2CP), nine 'scraped' (5VE/2YO/2CP) and 53 'silicone' teeth (5AN/2RM/2SP/44CP) at the ABC (Table 4; this table includes the teeth processed with multiple methods listed in Table 3), and 19 'scraped' (17RM/2FA) and 22 'silicone' teeth (8RM/10SP/4CP) from SBH (Table 5). This represents a total of 115 extractions on 108 different teeth. All extracts were tested for specific *Y. pestis* pesticin plasmid sequences and 71 were tested for *Y. pestis rpoB* chromosomal DNA. A summary of results for teeth from individuals tested in both laboratories is presented in Table 6. In both laboratories, no extraction blanks or negative controls yielded PCR products with *Y. pestis*-specific PCR primers. At SBH, a positive result was obtained from a tooth DNA extract using *pla* assay 'H' (see Table 2 and Table 5) when controls remained negative. Cloning and sequencing revealed that this band was identical to the engineered positive control fragment that contained a central indel compared with the native sequence. This sample was one of 12 samples which underwent pre-amplification at SBH as specified above. Pre-amplification was abandoned at SBH following this

Table 3. Direct comparison of the amplimers generated using four primer sets, from DNA extracts obtained using the 'silicone', 'scraped' and 'ground' extraction methods

Skeleton*	Site†	Tooth	Method‡	Primer set§			
				A	B	D	E
V1	VE	Molar	Scraped	−	m	−	M
			Ground	s	m	S	S
V10	VE	Molar	Scraped	−	m	S	−
			Ground	−	m	S	s
V4	VE	Molar	Scraped	−	m	S	S
			Ground	m	m	S	S
V3	VE	Molar	Scraped	s	−	S	M
			Ground	−	m	S	s
V7	VE	Molar	Scraped	−	−	−	−
			Ground	s	m	S	s
4245	YO	Molar	Scraped	−	m	S	−
			Ground	s	m	S	S
2654	YO	Molar	Scraped	M	m	S	S
			Ground	M	m	S	−
AR	CP	Molar	Scraped	−	m	m	−
			Ground	s	m	m	−
			Silicone	−	−	−	−
CG	CP	Molar	Ground	s	m	m	−
			Scraped	−	m	−	−
			Silicone	−	−	−	−

*Specimen from which teeth were extracted.

†Abbreviations as in Table 1.

‡Extraction method used to extract dentine prior to DNA extraction. Briefly, 'silicone' refers to samples encased in silicone rubber prior to dentine removal, following Gilbert *et al.* (2003b). 'Ground' refers to dentine recovery following complete grinding of specimens. 'Scraped' refers to longitudinal fracturing of teeth after which dentine was recovered from the pulp cavity by scraping with a dental pick (following Drancourt *et al.*, 1998).

§Primer pairs used for PCR. For abbreviation details refer to Table 2. s, Single PCR product amplified; S, single PCR product of expected size amplified; m, multiple PCR products amplified; M, multiple PCR products amplified, containing band of expected size; −, no PCR product amplified.

episode. At the ABC, five samples (4 'ground', 1 'scraped') yielded a single band of the expected size with *Y. pestis* pPst (pesticin plasmid)-specific primers. Four of these bands could be cloned and sequenced and none contained *Y. pestis*-specific sequence. No further bands were obtained with these primers at the ABC or SBH following adoption of the silicone-embedding technique. One tooth extracted by the 'silicone' method produced multiple bands in the *rpoB* PCR assay. The correct size band was cloned and the sequence of one clone contained a short match to *Y. pestis rpoB*, but this was found to represent a primer dimer of one of the PCR primers (Yprpob1).

Of four control samples at the ABC from the burial site not thought to contain plague victims (York), two (1 'ground', 1 'scraped') yielded PCR products with *Y. pestis*-specific primers but these products could not be cloned. No directly sequenced PCR products yielded readable DNA, indicating the presence of multiple DNA sequences of similar size in each amplified extract. Such products yielded up to 10 different sequences each when cloned at the ABC. No sequences were *Y. pestis*-specific DNA. All sequences obtained were searched using BLAST. The majority were reported as unknown, though some were loosely similar to other genera of bacteria. Within the clones of two samples amplified using 16S rDNA primers, single sequences matching, but not discriminating between, numerous proteobacteria including *Y. pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* were found. A data file containing all cloned bacterial DNA sequences and database matches is available in *Microbiology* Online (http://mic.sgmjournals.org).

Human PCR results

Human amplification products of the expected size were obtained in 53 out of 89 mitochondrial amplifications performed at the ABC, and in three out of 39 nuclear amplifications performed at SBH. No extraction blanks at the ABC yielded human DNA. Nevertheless, no attempt was made to sequence human products from samples extracted without silicone encasement at the ABC, due to the obvious bacterial, and therefore likely human sample, contamination. All human sequences are commonly found among Europeans and do not match any member of the ABC laboratory (data not shown). As this study has only a marginal interest in human DNA, the authenticity of the mitochondrial DNA amplified and cloned at the ABC has not been as exhaustively tested as would be appropriate in a study of human evolution. At SBH, nine out of 22 mitochondrial DNA amplifications yielded bands of the expected size. However, extraction blanks and the PCR control also yielded bands with this PCR. Cloning and sequencing of a contaminating band from one of the extraction controls and bands obtained from two teeth from different test subjects showed the contaminating band to be human mitochondrial DNA distinct both from that found in the two test subjects and also the individual who performed the DNA extraction and PCR.

At SBH no positive amplification results were obtained in the β-globin control PCR following adoption of the silicone-encasement technique and the use of a commercial kit for DNA extraction (specimens BT25–BT46). One microlitre of DNA extract was added to a control PCR for a TEM β-lactamase gene (Essack *et al.*, 2001) containing 4·3 pg of target DNA (PCR carried out in 25 µl volumes with Platinum *Taq* DNA polymerase as for the ABC standard method) to ensure that negative results in this assay and assays for *Y. pestis*-specific DNA were not due to PCR inhibitors in the DNA extracts. PCR inhibition was detected in two out of 22 tested extracts.

Table 4. Results of PCR amplification undertaken at the ABC

Table subheadings and abbreviations are as in Table 3 with the addition of NT, not tested. For PCR primer set abbreviation details refer to Table 2.

Skeleton	Site	Tooth	Method	Primer set						
				A	B	C	D	E	F	G
V2	VE	Molar	Ground	m	m	−	S†	s	S	NT
V8	VE	Molar	Ground	m	m	−	S†	S*	S	NT
V14	VE	Molar	Ground	m*	m	−	S†	−	S	NT
V9	VE	Molar	Ground	−	m	S†	−	s	S	NT
V1	VE	Molar	Scraped	−	m	−	−	M*	S	NT
V1	VE	Molar	Ground	s	m	−	S†	S‡	−	NT
V6	VE	Molar	Ground	−	m	−	S†	S*	S	NT
V10	VE	Molar	Scraped	−	m	−	S†	−	S	NT
V10	VE	Molar	Ground	−	m	s	S†	s†	S	NT
V12	VE	Molar	Ground	m	S	S†	S†	s†	−	NT
V4	VE	Molar	Scraped	−	m	S†	S†	S*	S	NT
V4	VE	Molar	Ground	m*	m	−	S†	S*	S	NT
V5	VE	Molar	Ground	M*	m	S†	S†	−	S	NT
V3	VE	Molar	Scraped	s	−	−	S†	M*	S	NT
V3	VE	Molar	Ground	−	m	−	S†	s	S	NT
V7	VE	Molar	Ground	s	m	S†	S†	s†	S	NT
V11	VE	Molar	Ground	M*	m	S†	S†	s†	S	NT
V13	VE	Molar	Ground	m	m	S†	S†	s†	S	NT
4245	YO	Molar	Scraped	−	m	S†	S†	−	S	NT
4245	YO	Molar	Ground	s	m	−	S†	S‡	−	NT
2654	YO	Molar	Scraped	M*	m	−	S†	S‡	S	NT
2654	YO	Molar	Ground	M*	m	−	S†	−	S	NT
V7	VE	Molar	Scraped	−	−	−	−	−	−	NT
S32_BK	CP	Molar	Silicone	M*	NT	NT	S*	−	NT	S
AE	CP	Molar	Silicone	−	NT	NT	−	−	NT	−
XA	CP	Molar§	Silicone	−	NT	NT	−	−	NT	S
BN	CP	Molar§	Silicone	−	NT	NT	−	−	NT	−
CR	CP	Molar	Silicone	−	NT	NT	−	−	NT	−
CP	CP	Molar§	Silicone	−	NT	NT	−	−	NT	−
CV	CP	Molar	Silicone	−	NT	NT	S*	−	NT	−
AD	CP	Molar	Silicone	−	NT	NT	−	−	NT	−
BN	CP	Molar	Silicone	−	NT	NT	S*	−	NT	−
DF	CP	Molar	Silicone	−	NT	NT	−	−	NT	−
DB	CP	Molar	Silicone	M*	NT	NT	S*	−	NT	S
XA	CP	Molar§	Silicone	−	NT	NT	S*	−	NT	S*
BT	CP	Molar	Silicone	−	NT	NT	−	−	NT	S
BR	CP	Premolar	Silicone	−	NT	NT	−	−	NT	S
AO	CP	Molar§	Silicone	−	NT	NT	−	−	NT	S
AP	CP	Molar	Silicone	−	NT	NT	−	−	NT	S
CY	CP	Molar	Silicone	−	NT	NT	−	−	NT	S
S34_L	CP	Molar	Silicone	−	NT	NT	−	−	NT	S
BN	CP	Molar§	Silicone	−	NT	NT	−	−	NT	−
CH	CP	Molar	Silicone	−	NT	NT	−	−	NT	S
AI	CP	Molar	Silicone	−	NT	NT	−	−	NT	S
CA	CP	Molar	Silicone	−	NT	NT	−	−	NT	S
DE	CP	Molar	Silicone	−	NT	NT	−	−	NT	S
AS	CP	Molar	Silicone	−	NT	NT	−	−	NT	−
CM	CP	Molar	Silicone	−	NT	NT	−	−	NT	S
XA	CP	Molar	Silicone	−	NT	NT	−	−	NT	S
S60a	CP	Molar	Silicone	−	NT	NT	−	−	NT	−

Table 4. cont.

Skeleton	Site	Tooth	Method	Primer set						
				A	B	C	D	E	F	G
XC	CP	Molar	Silicone	–	NT	NT	–	–	NT	S
CI	CP	Molar	Silicone	–	NT	NT	–	–	NT	S*
CK	CP	Molar	Silicone	–	NT	NT	–	–	NT	–
AH	CP	Molar	Silicone	–	NT	NT	–	–	NT	S*
S60b	CP	Molar	Silicone	–	NT	NT	–	–	NT	S
BU	CP	Molar§	Silicone	–	NT	NT	–	–	NT	S
AO	CP	Molar§	Silicone	–	NT	NT	–	–	NT	–
XD	CP	Premolar	Silicone	–	NT	NT	–	–	NT	S*
BU	CP	Molar	Silicone	–	NT	NT	–	–	NT	S
BN	CP	Molar§	Silicone	–	NT	NT	–	–	NT	s†
BU	CP	Molar§	Silicone	–	NT	NT	–	–	NT	s†
6181	RM	Molar	Silicone	–	NT	NT	–	–	NT	s†
3780	SP	Molar	Silicone	–	NT	NT	–	–	NT	s†
3817	SP	Molar	Silicone	–	NT	NT	–	–	NT	s†
9830	RM	Molar	Silicone	–	NT	NT	–	–	NT	–
C1	AN	Premolar	Silicone	NT	NT	NT	NT	–	NT	–
C1	AN	Premolar	Silicone	NT	NT	NT	NT	–	NT	–
C1	AN	Premolar	Silicone	NT	NT	NT	NT	–	NT	–
C1	AN	Canine	Silicone	NT	NT	NT	NT	–	NT	–
C1	AN	Premolar	Silicone	NT	NT	NT	NT	–	NT	–
AR	CP	Molar	Silicone	–	NT	NT	NT	–	NT	S†
CG	CP	Molar	Silicone	–	NT	NT	NT	–	NT	–
CB	CP	Molar	Silicone	–	NT	NT	NT	–	NT	–
CN	CP	Molar	Silicone	–	NT	NT	NT	–	NT	–
AR	CP	Molar	Scraped	–	m	NT	m	–	NT	NT
AR	CP	Molar	Ground	s	m	NT	m	–	NT	NT
CG	CP	Molar	Ground	s	m	NT	m	–	NT	NT
CG	CP	Molar	Scraped	–	m	NT	–	–	NT	NT
AR	CP	Molar	Silicone	–	–	NT	–	–	NT	NT
CG	CP	Molar	Silicone	–	–	NT	–	–	NT	NT

*Denotes amplicons that were cloned and sequenced.
†Denotes amplicons that were sequenced directly.
‡Denotes amplicons that could not be cloned.
§Denotes unerupted molar.

DISCUSSION

Previous extraction techniques are unsuited to preventing bacterial contamination of the DNA extract

Almost all previous work that has employed teeth as a source of aDNA has used either the 'ground' (cf. Oota *et al.*, 1995) or the 'scraped' (cf. Drancourt *et al.*, 1998; Raoult *et al.*, 2000) method described to recover dentine. Dental enamel, as a highly mineralized material, is extremely resistant to diagenesis, but may be permeable to contaminating environmental DNA. The extractions performed here on 'ground' samples, where DNA from environmental sources was amplified, despite UV light and bleach pretreatment, suggest that contamination persists within external layers of teeth despite external decontamination. Our extractions of 'scraped' teeth meanwhile point to the relative ease with which these contaminants may subsequently enter the DNA extraction, even when high levels of preventative care are taken. A plausible route is via contact with gloves, when the teeth are manipulated. Encasing teeth in silicone appears to act as a barrier to such movement and may explain the observed reduction in contamination levels with this method.

Absence of authentic *Y. pestis* DNA in the samples

No evidence for surviving *Y. pestis* DNA was found in this study, despite the examination of a large number of samples from five mass graves, including two well-documented

Table 5. Results of PCR amplification undertaken at SBH

Headings and notation as in Tables 2, 3 and 4, with the exception of those shown in the footnotes.

Sample*	Skeleton	Site	Tooth	Method	PCR primer pair					
					E	G	H	I	J	K
BT1/A1	11314	RM	Molar	Scraped	NT	NT	NT	–	NT	–
BT2/A2	11314	RM	Molar	Scraped	NT	NT	NT	–	NT	S
BT4/M1	1533	FA	Molar	Scraped	NT	NT	NT	–	NT	–
BT6/A3	11143	RM	Canine	Scraped	NT	NT	NT	–	NT	–
BT8/A4	11314	RM	Incisor	Scraped	NT	NT	NT	–	NT	–
BT9/M2	1533	FA	Canine	Scraped	NT	NT	NT	–	NT	–
BT10/A5	11314	RM	Incisor	Scraped	NT	NT	NT	–	NT	–
BT12/A6	11034	RM	Molar	Scraped†	NT	NT	–	NT	NT	–
BT13/A7	11034	RM	Molar	Scraped†	NT	NT	–	NT	NT	–
BT14/A8	11034	RM	Incisor	Scraped†	NT	NT	–	NT	NT	S
BT15/A9	9830	RM	Molar	Scraped†	NT	NT	M‡§	NT	NT	S
BT17/A10	9830	RM	Molar	Scraped†	NT	NT	–	NT	NT	–
BT18/A11	5383	RM	Molar	Scraped†	NT	NT	–	NT	NT	–
BT19/A12	11034	RM	Molar	Scraped†	NT	NT	–	NT	NT	–
BT20/A13	9830	RM	Molar	Scraped†	NT	NT	–	NT	NT	–
BT21/A14	6181	RM	Molar	Scraped†	NT	NT	–	NT	NT	–
BT22/A15	9830	RM	Molar	Scraped†	NT	NT	–	NT	NT	–
BT23/A16	6181	RM	Molar	Scraped†	NT	NT	–	NT	NT	–
BT24/A17	8440	RM	Molar	Scraped†	NT	NT	–	NT	NT	–
BT25/A18	9859	RM	Molar	Silicone	NT	s	NT	NT	–	NT
BT26/A19	7440	RM	Molar	Silicone	NT	s	NT	NT	–	NT
BT27/A20	19009	SP	Molar	Silicone	NT	–	NT	NT	–	NT
BT28/A21	3780	SP	Molar	Silicone	NT	–	NT	NT	–	NT
BT29/A22	11039	RM	Incisor	Silicone	NT	–	NT	NT	–	NT
BT39/A23	9830	RM	Incisor	Silicone	NT	s	NT	NT	–	NT
BT31/A24	3816	SP	Molar	Silicone	NT	–	NT	NT	–	NT
BT32/A25	3815	SP	Incisor	Silicone	NT	–	NT	NT	–	NT
BT33/A26	5383	RM	Molar	Silicone	NT	–	NT	NT	–	NT
BT34/A27	3817	SP	Molar	Silicone	NT	–	NT	NT	–	NT
BT35/A28	5383	RM	Molar	Silicone	–	–	NT	NT	–	–
BT36/A29	9830	RM	Incisor	Silicone	–	S	NT	NT	–	–
BT37/A30	19009	SP	Molar	Silicone	–	–	NT	NT	–	–
BT38/A31	3816	SP	Molar	Silicone	–	M	NT	NT	–	–
BT39/A32	3817	SP	Molar	Silicone	–	–	NT	NT	–	–
BT40/A33	6181	RM	Incisor	Silicone	–	M‡	NT	NT	–	–
BT41/A34	3780	SP	Incisor	Silicone	–	–	NT	NT	–	–
BT42/A35	3815	SP	Premolar	Silicone	–	–	NT	NT	–	–
BT43/A36	BR	CP	Incisor	Silicone	–	S	NT	NT	–	–
BT44/A37	CB1	CP	Premolar	Silicone	–	M	NT	NT	–	–
BT45/A38	AR	CP	Premolar	Silicone	–	M‡	NT	NT	–	–
BT46/A39	AE	CP	Premolar	Silicone	–	–	NT	NT	–	–

*DNA extraction name.

†Samples extracted with guanidinium isocyanate/silica method.

‡PCR products cloned and sequenced.

§Identified as contaminated with PCR-positive control DNA.

plague pits and several other probable plague-victim burial sites. This result strongly contrasts with previous studies (Drancourt *et al.*, 1998; Raoult *et al.*, 2000). PCR amplification using *Y. pestis*-specific primers did produce amplicons, though not of *Y. pestis* DNA. Similarly, cloned amplicons from the non-specific bacterial 16S rDNA revealed sequences that matched a variety of bacteria. While two cloned 16S rDNA sequence fragments obtained with one set of

Table 6. Summary of presence (+) and absence (−) of amplifiable *Y. pestis* DNA within teeth DNA extracts taken from skeletons investigated by both research groups

Headings and abbreviations as in Tables 2 and 3, with the addition of those shown in the footnotes.

Skeleton	Primer set						
	H*	I*	J*	E*	E†	D†	A†
6181	−	NT	−	−	−	NT	NT
3780	−	NT	−	−	−	NT	NT
3817	−	NT	−	−	−	NT	NT
9830	+‡	NT	−	−	−	NT	NT
BR	NT	−	−	−	−	−	−
CB1	NT	−	−	−	−	−	−
AR	NT	−	−	−	−	−	−
AE	NT	−	−	−	−	−	−

*Primer set used at SBH.

†Primer set used at the ABC.

‡Positive control contaminant amplified from one extract (three other teeth from the same individual tested negative with the same primers).

primers resembled *Y. pestis*, and although this short amplicon incorporates a variable loop that can be used to distinguish species of bacteria, the sequence was shared by at least seven different prokaryotes belonging to three genera found on the Ribosomal Database Project II web site (http://rdp.cme.msu.edu/html/) and numerous other bacterial sequences in the NCBI BLAST database (http://www.ncbi.nlm.nih.gov/blast/). The database matches of most 16S rDNA sequences obtained suggest the contaminating DNA probably originates in contemporary soil bacteria, rather than authentic aDNA sources (see supplementary data). Evidence to support this hypothesis includes the lack of *post-mortem* damage-driven sequence variation observed among multiple clones of the same species (Pääbo, 1989), the large size of fragments obtained (Pääbo, 1989) and, most importantly, the presence of micro-organism DNA where human DNA cannot be amplified.

The human DNA bears characteristics that suggest authenticity, including the observed consistency of amplified sequences and the observed spectrum of damage within the human cloned sequences (Pääbo, 1989; Gilbert *et al.*, 2003a), the pattern of nuclear to mitochondrial DNA survival (Hofreiter *et al.*, 2001) and the appropriate behaviour of negative controls and extraction blanks (Cooper & Poinar, 2000). However, it is very difficult to guarantee the authenticity of aDNA from human samples (cf. Handt *et al.*, 1996; Cooper, 1997; Kolman & Tuross, 2000; Hofreiter *et al.*, 2001), thus it is possible that the human results are derived from contaminant DNA. Such a case would add support to the conclusion that the amplified bacterial DNA is from modern environmental contaminants.

In contrast to our findings, previous studies reported successful direct sequencing of *Y. pestis*-specific PCR products (Drancourt *et al.*, 1998; Raoult *et al.*, 2000) from ancient teeth. This implies a low level of contaminating non-*Y. pestis* bacterial DNA, despite using a dentine extraction method that this study has demonstrated to be contamination-prone. Two questions therefore need to be answered. Firstly, why such levels of contaminating DNA from bacteria other than *Y. pestis* are found in our study, even when *Y. pestis* specific primers, high-fidelity enzymes, dedicated aDNA facilities, rigorous cleaning and established extraction techniques are used. Secondly, why it was not possible to amplify *Y. pestis*-specific DNA from samples of plague victims that yield what appears to be authentic human DNA.

A tempting explanation for the discrepancy between the results is heterogeneity between archaeological sites. Samples studied in this work were obtained from north-western European locations, which differ environmentally from the relatively warmer and drier southern French locations of the previous studies. It is possible, therefore, that the diagenetic conditions at the southern locations were conducive to ancient *Y. pestis* survival and that local environmental bacteria do not share amplifiable DNA similarities with *Y. pestis*. Several possible flaws can be identified with this hypothesis. aDNA studies have repeatedly demonstrated that an inverse correlation exists between average temperature of archaeological site, humidity and aDNA retrieval (Höss *et al.*, 1996). It is surprising that samples used in the two successful studies were from warmer locations than those used here. An alternative environmental variable to consider is that the samples analysed in this study can be expected to have experienced more groundwater. Although this has not been directly implicated in aDNA survival, Nielsen-Marsh & Hedges (2000) note an inverse correlation of sample exposure to water and aDNA survival. However, the ability to amplify what appears to be endogenous human aDNA from extracts suggests host DNA survival is not an issue. The reduced rate of amplification of DNA associated with environmental bacteria from the pulp of silicone-encased and drilled teeth compared with glove-handled split teeth suggests that the pulp is indeed relatively protected from environmental contamination, whether arising due to water penetration while in the soil or as a result of handling during processing. The wetter northern environments may harbour a specific range of micro-organisms that share amplifiable similarities with *Y. pestis*. However, with increasing numbers of scientists suggesting global dispersion of micro-organisms (Finlay & Clarke, 1999; Finlay *et al.*, 1999), it appears strange the two (globally close) environments should differ so much in micro-organism content.

A further explanation is that the individuals from whom the samples derive were either infected by a *Y. pestis* strain lacking the plasmid-located sites for amplification or not infected with *Y. pestis* (because they were not victims of the

Black Death, or because the infection did not seed the pulp cavity, or because the Black Death and subsequent plagues were not caused by *Y. pestis*). The first hypothesis is unlikely, as although some *Y. pestis* plasmids may vary between strains, the plasmid containing the *pla* gene is a consistent feature of contemporary *Y. pestis* isolates (Filippov *et al.*, 1990). In addition, a *Y. pestis* chromosomal target (*rpoB*) was employed for the ABC-processed teeth. If the 66 selected individuals were not infected by *Y. pestis* but were surrounded by infected individuals, then this would be a most unusual sampling error. The second hypothesis is plausible. There is no guarantee that bacteria causing a systemic infection entered the teeth of infected individuals, even if individuals were infected with *Y. pestis*. Contemporary microbiological studies with dental pulp (primarily but not exclusively in the context of dental caries) are able to both directly culture and obtain DNA by PCR from various anaerobes and streptococci (Hoshino *et al.*, 1992; Conrads *et al.*, 1997; Bate *et al.*, 2000), i.e. typical oral bacteria. It has been demonstrated that DNA specific to *Coxiella burnetii* could be detected in dental pulp of five out of 10 guinea pigs 15–20 days after intraperitoneal injection with the bacteria (Aboudharam *et al.*, 2000). However, in no animal were blood cultures and dental pulp PCR positive at the same time. Consequently, the relationship between bacteraemia and pulp colonization is not straightforward and it is possible that *Y. pestis* may not have been present in the teeth specimens but *Y. pestis* infection still caused death.

The third hypothesis – that the Black Death was not caused by *Y. pestis* – is controversial (Scott & Duncan, 2001; Cohn, 2002), but cannot be immediately discounted. Recent re-examinations of the epidemiology of the Black Death from contemporary descriptions and mortality records have suggested that it does not correspond to the illness referred to as bubonic plague and that the organism responsible was therefore not *Y. pestis* (Scott & Duncan, 2001; Cohn, 2002). These authors argue that instead of the cumbersome rat–flea vector system of *Y. pestis* infections, a more direct person-to-person spread of the disease is required to cause the predominantly city-based outbreaks with subsequent rapid spread over long distances. They also postulate that the illness itself, presenting multiple haemorrhagic skin lesions (tokens) and killing 40–50 % of the population, does not convincingly resemble microbiologically confirmed bubonic plague in the pre-antibiotic era. The proponents of these hypotheses regard the molecular evidence of the Marseille group as either relevant only to distinct local outbreaks of bubonic plague in Marseille and other parts of Provence (Scott & Duncan, 2001) or in need of independent corroboration (Cohn, 2002). Scott & Duncan (2001) instead suggest that the Black Death was a form of viral haemorrhagic fever, a group of emerging infections caused predominantly by RNA Filoviruses (Khan *et al.*, 1998). If so, they would be impossible to identify by current techniques on available remains from Black Death victims [viral RNA extraction and successful RT-PCR from historical specimens has only been recorded from frozen or formalin-preserved organs (Taubenberger *et al.*, 1997; Reid *et al.*, 1999; Basler *et al.*, 2001)].

In contrast to our findings, all but one individual tested from three different locations in southern France in the previous studies yielded positive PCR amplifications of fragments of the *Y. pestis pla* or *rpoB* genes (Drancourt *et al.*, 1998; Raoult *et al.*, 2000). The authors also report in their second publication a higher percentage of the older erupted teeth to be positive for *Y. pestis*-specific DNA (20 out of 23 teeth positive) than the more recent unerupted teeth used in the first publication (8 out of 13 teeth positive). This pattern of results seems surprising for several reasons. Firstly, during an epidemic, not all deaths can be expected to arise due to the specific pathogen (Kiple, 1993). Thus, as increasing numbers of specimens are examined, the chance of analysing authentic uninfected remains increases. Therefore, it seems unlikely that, among the eight individuals sampled in these two studies, only one negative for *Y. pestis* DNA was observed. Secondly, preservation of *Y. pestis* DNA in nearly all teeth from infected individuals is unlikely due to the nature of DNA degradation. Pfeiffer *et al.* (1999) observed that storage of teeth in soil for only 6 weeks leads to a decrease in extractable endogenous DNA by 90 %. Therefore, aDNA tests for bacterial infection can be expected to demonstrate a proportion of false-negative results. For example, independently replicated *M. tuberculosis* aDNA assays on comparably old specimens that display characteristic diagnostic bony lesions did not yield 100 % positive results (Haas *et al.*, 2000) despite the robust successfulness of assays for mycobacterial aDNA in various laboratories (Zink *et al.*, 2001; Haas *et al.*, 2000; Rothschild *et al.*, 2001; Spigelman *et al.*, 2002; Fletcher *et al.*, 2003a, b). We have found no published data on long-term preservation of aDNA from *Enterobacteriaceae* in human specimens, other than the Marseille group's publications on *Y. pestis*. Evidence of rapid *Y. pestis* DNA degradation over short periods of time is provided in the article from which the original *pla* PCR assay was developed [an assay for *Y. pestis* in infected fleas (Hinnebusch & Schwan, 1993)]. After 5 months storage, sensitivity of the original assay dropped from 100 to 90 % in fleas stored at −20 °C, and to 55 % in fleas stored at room temperature in ethanol (Hinnebusch & Schwan, 1993). Although the target in this original assay was larger (478 bp) than the 300 and 150 bp targets used by ourselves and the Marseille group, *pla* target aDNA preservation over five centuries in soil at Mediterranean ambient temperatures would have to be remarkably better than this to yield the positivity rate of 20 out of 23 14th century teeth that they describe (Raoult *et al.*, 2000). Another factor suggesting excellent DNA preservation in southern France is the detection of *rpoB* in the first publication (Drancourt *et al.*, 1998), albeit by two rounds of PCR. One of the reasons *pla* is used as a target for current *Y. pestis* detection assays is the high copy number of the pPst (pesticin) plasmid on which it is located – over 100 per bacterium (Parkhill *et al.*, 2001). In contrast, *rpoB* is a

single copy chromosomal gene (Parkhill *et al.*, 2001), a relatively poorly represented target which is not used as the basis for any current assays for *Y. pestis* pre-culture diagnosis.

Poor laboratory technique may explain our results, as could differences in the methodologies used here and previously. However, similar results are found by two independent laboratories, one of which is a facility dedicated to aDNA research and has published numerous replicated aDNA studies (e.g. Cooper *et al.*, 2001; Shapiro *et al.*, 2002; Barnes *et al.*, 2002; Endicott *et al.*, 2003; Gilbert *et al.*, 2003a, b). Secondly, the lack of contaminant DNA sequence variation observed between samples from one archaeological site and the much larger sequence variation between different sites points to the presence of contaminants in samples prior to extraction. Lastly, the techniques used here include the use of the modified PCR enzyme Platinum *Taq* Hifidelity (as opposed to 'standard' *Taq* polymerases). The increased yields and successes of aDNA amplifications performed using this enzyme have been noted previously (Willerslev *et al.*, 1999; Hansen *et al.*, 2001; Gilbert *et al.*, 2003a) especially in amplifying low-copy-number DNA.

It is possible that the DNA sequences presented previously (Drancourt *et al.*, 1998; Raoult *et al.*, 2000) derive from the contamination of DNA extracts with formerly amplified or extracted *Y. pestis* DNA. Other examples exist of pioneering bacterial aDNA studies that could not be replicated and may have resulted from unsuspected contamination. Christner *et al.* (2000) reported unsuccessful attempts at replicating aDNA detection from bacteria within Greenland ice cores (Catranis & Starmer, 1991; Ma *et al.*, 1999). Graur & Pupko (2001) and Nickle *et al.* (2002) have been unable to replicate detection of the 250 million-year-old halotolerant bacterium of Vreeland *et al.* (2000).

The previous *Y. pestis* aDNA studies were undertaken in a busy facility not dedicated to aDNA work, where numerous other bacteriological studies are undertaken, and initially using a positive control of modern *Y. pestis* DNA, all of which are risk factors for contamination. However, occurrence of contamination in aDNA work even under the most stringent conditions is well documented (Handt *et al.*, 1996; Kolman, 1999; Kolman & Tuross, 2000; Hofreiter *et al.*, 2001). In our own study, the use of a modified positive control in the SBH laboratory (a non-dedicated aDNA set-up comparable to that used by the Marseille group) has resulted in contamination problems with bacterial DNA. This clear occurrence of selective contamination where, importantly, negative controls remained blank is especially revealing. This phenomenon, termed the 'carrier effect', and the dangers of relying on negative controls are discussed elsewhere (Cooper, 1994; Handt *et al.*, 1994; Kolman, 1999). Raoult *et al.* (2000) developed 'suicide PCR' to avoid the possibility of amplicon contamination, but this technique is not resilient to contamination by fragments of extracted modern DNA, from any organism sharing the DNA sequence of interest.

Conclusion

This study has failed to replicate previous reports of specific *Y. pestis* DNA amplification from dental pulp residues extracted from historical plague victims. Analyses of 16S rDNA PCR products reveal a wide variety of bacterial DNA in the extracts from teeth prepared using the previous method. An improved method has been developed for pulp-cavity sampling, which resulted in a reduced number of amplicons from apparent environmental contaminants, and it is recommended that future studies consider adopting the approach, which is cheap, simple and effective.

It is difficult to interpret the previous reports of almost uniform positive results, when similar techniques reveal the non-specificity of supposedly *Y. pestis*-specific primers in this application, the ease of positive control cross-contamination and the apparent lack of *Y. pestis* DNA in samples that yield human DNA, from well-documented archaeological plague sites. Only a minority of published ancient pathogen DNA studies clearly indicate that they have involved independent replication by separate laboratories. Most of the replicated reports concern successful amplification of *M. tuberculosis* from skeletal remains or mummified tissue (Zink *et al.*, 2001; Haas *et al.*, 2000; Rothschild *et al.*, 2001; Spigelman *et al.*, 2002; Fletcher *et al.*, 2003a, b). The special properties of *M. tuberculosis* that enable lengthy persistence in the body as a latent infection have been suggested to preserve its DNA after the death of its host (Fletcher *et al.*, 2003a). There is no evidence that *Y. pestis* can cause persistent latent disease in humans. Independent replication seems particularly necessary when attempting novel retrospective diagnosis of diseases that cause rapid death and leave no specific bony changes. Authoritative aDNA evidence offers the only conclusive method to match current pathogens with ancient epidemics of disease, but misleading DNA data are very easily generated from contemporary bacteria. For these reasons we believe that until an independently replicated, successful study on *Y. pestis* aDNA is undertaken in a suitable, controlled environment, meeting best practice guidelines in aDNA research (Cooper & Poinar, 2000), it is premature to claim (Drancourt & Raoult, 2002; Raoult & Drancourt, 2002) that aDNA studies have unequivocally proved *Y. pestis* to be the cause of the Black Death and subsequent historical plagues. The aetiology of one of the major pandemics of the last millennium remains unproven by molecular techniques.

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REFERENCES

Aboudharam, G., La Scola, B., Raoult, D. & Drancourt, M. (2000). Detection of *Coxiella burnetii* in the dental pulp during experimental bacteremia. *Microb Pathog* **28**, 249–254.

Austin, J. J., Ross, A. J., Smith, A. B., Fortey, R. A. & Thomas, R. H. (1997). Problems of reproducibility – does geologically ancient DNA survive in amber-preserved insects? *Proc R Soc Lond B Biol Sci* **264**, 467–474.

Barnes, I., Matheus, P., Shapiro, B., Jensen, D. & Cooper, A. (2002). Dynamics of mammal population extinctions in Eastern Beringia during the last glaciation. *Science* **295**, 2267–2270.

Basler, C. F., Reid, A. H., Dybing, J. K. & 9 other authors (2001). Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. *Proc Natl Acad Sci U S A* **98**, 2746–2751.

Bate, A. L., Ma, J. K. & Pitt Ford, T. R. (2000). Detection of bacterial virulence genes associated with infective endocarditis in infected root canals. *Int Endod J* **33**, 194–203.

Boom, R., Sol, C. J., Salimans, M. M., Jansen, C. L., Wertheim-van Dillen, P. M. & van der Noordaa, J. (1990). Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* **28**, 495–503.

Catranis, C. & Starmer, W. T. (1991). Microorganisms entrapped in glacial ice. *Antarct J U S* **26**, 324–326.

Christner, B. C., Mosley-Thompson, E., Thompson, L. G., Zagorodnov, V., Sandman, K. & Reeve, J. N. (2000). Recovery and identification of viable bacteria immured in glacial ice. *Icarus* **144**, 479–485.

Cohn, S. (2002). *The Black Death Transformed: Disease and Culture in Early Renaissance Europe.* London: Arnold.

Conrads, G., Gharbia, S. E., Gulabivala, K., Lampert, F. & Shah, H. N. (1997). The use of a 16S rDNA directed PCR for the detection of endodontopathogenic bacteria. *J Endod* **23**, 433–438.

Cooper, A. (1994). DNA from museum specimens. In *Ancient DNA*, pp. 149–165. Edited by B. Hermann & S. Hummel. New York: Springer-Verlag.

Cooper, A. (1997). Reply to Stoneking: ancient DNA – how do you really known when you have it? *Am J Hum Genet* **60**, 1001–1002.

Cooper, A. & Poinar, H. N. (2000). Ancient DNA: do it right or not at all. *Science* **289**, 1139.

Cooper, A., Lalueza-Fox, C., Anderson, S., Rambaut, A., Austin, J. & Ward, R. (2001). Complete mitochondrial genome of two extinct moas clarify ratite evolution. *Nature* **409**, 704–707.

Deng, W., Burland, V., Plunkett, G., 3rd & 18 other authors (2002). Genome sequence of *Yersinia pestis* KIM. *J Bacteriol* **184**, 4601–4611.

Drancourt, M. & Raoult, D. (2002). Molecular insights into the history of plague. *Microbes Infect* **4**, 105–109.

Drancourt, M., Aboudharam, G., Signoli, M., Dutour, O. & Raoult, D. (1998). Detection of 400-year-old *Yersinia pestis* DNA in human dental pulp: an approach to the diagnosis of ancient septicemia. *Proc Natl Acad Sci U S A* **95**, 12637–12640.

Endicott, P., Gilbert, M. T., Stringer, C., Lalueza-Fox, C., Willerslev, E., Hansen, A. J. & Cooper, A. (2003). The genetic origins of the Andaman Islanders. *Am J Hum Genet* **72**, 178–184.

Essack, S. Y., Hall, L. M., Pillay, D. G., McFayden, M. L. & Livermore, D. M. (2001). Complexity and diversity of *Klebsiella pneumoniae* strains with extended-spectrum beta-lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. *Antimicrob Agents Chemother* **45**, 88–95.

Filippov, A. A., Solodovnikov, N. S., Kookleva, L. M. & Protsenko, O. A. (1990). Plasmid content in *Yersinia pestis* strains of different origin. *FEMS Microbiol Lett* **55**, 45–48.

Finlay, B. J. & Clarke, K. J. (1999). Ubiquitous dispersal of microbial species. *Nature* **400**, 828.

Finlay, B. J., Esteban, G. F., Olmo, J. L. & Tyler, P. A. (1999). Global distribution of free-living microbial species. *Ecography* **22**, 138–144.

Fletcher, H. A., Donoghue, H. D., Holton, J., Pap, I. & Spigelman, M. (2003a). Widespread occurrence of *Mycobacterium tuberculosis* DNA from 18th–19th century Hungarians. *Am J Phys Anthropol* **120**, 144–152.

Fletcher, H. A., Donoghue, H. D., Taylor, G. M., van der Zanden, A. G. & Spigelman, M. (2003b). Molecular analysis of *Mycobacterium tuberculosis* DNA from a family of 18th century Hungarians. *Microbiology* **149**, 143–151.

Gilbert, M. T. P., Hansen, A. J., Willerslev, E., Rudbeck, L., Barnes, I., Lynnerup, N. & Cooper, A. (2003a). Characterisation of genetic miscoding lesions caused by post mortem damage. *Am J Hum Genet* **72**, 48–61.

Gilbert, M. T. P., Willerslev, E., Hansen, A. J., Rudbeck, L., Barnes, I., Lynnerup, N. & Cooper, A. (2003b). Distribution patterns of post mortem damage in human mitochondrial DNA. *Am J Hum Genet* **72**, 32–47.

Graur, D. & Pupko, T. (2001). The Permian bacterium that isn't. *Mol Biol Evol* **18**, 1143–1146.

Greer, C. E., Peterson, S. L., Kiviat, N. B. & Manos, M. M. (1991). PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time. *Am J Clin Pathol* **95**, 117–124.

Gutierrez, G. & Marin, A. (1998). The most ancient DNA recovered from an amber-preserved specimen may not be as ancient as it seems. *Mol Biol Evol* **15**, 926–929.

Haas, C. J., Zink, A., Molnar, E. & 7 other authors (2000). Molecular evidence for different stages of tuberculosis in ancient bone samples from Hungary. *Am J Phys Anthropol* **113**, 293–304.

Handt, O., Hoss, M., Krings, M. & Paabo, S. (1994). Ancient DNA: methodological challenges. *Experientia* **50**, 524–529.

Handt, O., Krings, M., Ward, R. & Pääbo, S. (1996). The retrieval of ancient human DNA sequences. *Am J Hum Genet* **59**, 368–376.

Hansen, A., Willerslev, E., Wiuf, C., Mourier, T. & Arctander, P. (2001). Statistical evidence for miscoding lesions in ancient DNA templates. *Mol Biol Evol* **18**, 262–265.

Hawkins, D. (1990). The Black Death and the new London cemeteries of 1348. *Antiquity* **60**, 637–642.

Hinnebusch, J. & Schwan, T. G. (1993). New method for plague surveillance using polymerase chain reaction to detect *Yersinia pestis* in fleas. *J Clin Microbiol* **31**, 1511–1514.

Hofreiter, M., Serre, D., Poinar, H. N., Kuch, M. & Pääbo, S. (2001). Ancient DNA. *Nat Rev Genet* **2**, 353–359.

Hoshino, E., Naomi, A., Michiko, S. & Kohichi, K. (1992). Bacterial invasion of non-exposed dental pulp. *Int Endod J* **25**, 2–5.

Höss, M., Jaruga, P., Zastawny, T. H., Dizdaroglu, M. & Pääbo, S. (1996). DNA damage and DNA sequence retrieval from ancient tissues. *Nucleic Acids Res* **24**, 1304–1307.

Khan, A. S., Sanchez, A. & Pflieger, A. K. (1998). Filoviral haemorrhagic fevers. *Br Med Bull* **54**, 675–692.

Kiple, K. F. (1993). *The Cambridge World History of Human Disease.* Cambridge: Cambridge University Press.

Kolman, C. J. (1999). Molecular anthropology – progress and perspectives on ancient DNA technology. In *Genomic Diversity: Applications in Human Population Genetics.* Edited by S. S. Papiha & R. Deka. New York: Academic/Plenum Publishers.

Kolman, C. J. & Tuross, N. (2000). Ancient DNA analysis of human populations. *Am J Phys Anthropol* **111**, 5–23.

Lynnerup, N. (1992). *Anthropological Report on Human Remains from Vodroffsgaard, Copenhagen, AS 3/92.* On file at the Laboratory of Biological Anthropology, University of Copenhagen, Copenhagen, Denmark.

Ma, L.-J., Rogers, S. O., Catranis, C. M. & Starmer, W. T. (1999). Detection and characterization of ancient fungi entrapped in glacial ice. *Mycologia* **92**, 286–295.

Nerlich, A. G., Haas, C. J., Zink, A., Szeimies, U. & Hagedorn, H. G. (1997). Molecular evidence for tuberculosis in an ancient Egyptian mummy. *Lancet* **350**, 1404.

Nickle, D. C., Learn, G. H., Rain, M. W., Mullins, J. I. & Mittler, J. E. (2002). Curiously modern DNA from a ''250 million-year-old'' bacterium. *J Mol Evol* **54**, 134–137.

Nielsen-Marsh, C. & Hedges, R. E. M. (2000). Patterns of diagenesis in bone I: effects of site environments. *J Archaeol Sci* **27**, 1139–1150.

Oota, H., Saitou, N., Matsushita, T. & Ueda, S. (1995). A genetic study of 2,000-year-old human remains from Japan using mitochondrial DNA sequences. *Am J Phys Anthropol* **98**, 133–145.

Pääbo, S. (1989). Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. *Proc Natl Acad Sci U S A* **86**, 1939–1943.

Parkhill, J., Wren, B. W., Thomson, N. R. & 32 other authors (2001). Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413**, 523–527.

Pfeiffer, H., Huhne, J., Seitz, B. & Brinkmann, B. (1999). Influence of soil storage and exposure period on DNA recovery from teeth. *Int J Legal Med* **112**, 142–144.

Rafi, A., Spigelman, M., Stanford, J., Lemma, E., Donoghue, H. & Zias, J. (1994). *Mycobacterium leprae* DNA from ancient bone detected by PCR. *Lancet* **343**, 1360–1361.

Raoult, D. & Drancourt, M. (2002). Cause of Black Death. *Lancet Infect Dis* **2**, 459.

Raoult, D., Aboudharam, G., Crubezy, E., Larrouy, G., Ludes, B. & Drancourt, M. (2000). Molecular identification by ''suicide PCR'' of *Yersinia pestis* as the agent of medieval black death. *Proc Natl Acad Sci U S A* **97**, 12800–12803.

Reid, A. H., Fanning, T. G., Hultin, J. V. & Taubenberger, J. K. (1999). Origin and evolution of the 1918 ''Spanish'' influenza virus hemagglutinin gene. *Proc Natl Acad Sci U S A* **96**, 1651–1656.

Richards, M., Sykes, B. & Hedges, R. (1995). Authenticating DNA extracted from ancient skeletal remains. *J Archaeol Sci* **22**, 291–299.

Ringboel Bitsch, B. (1991). *Archaeological Excavation Report ''Vodroffsgaard'', KBM 836.* On file at Copenhagen City Museum, Copenhagen, Denmark.

Rothschild, B. M., Martin, L. D., Lev, G., Bercovier, H., Bar-Gal, G. K., Greenblatt, C., Donoghue, H., Spigelman, M. & Brittain, D. (2001). *Mycobacterium tuberculosis* complex DNA from an extinct bison dated 17,000 years before the present. *Clin Infect Dis* **33**, 305–311.

Salo, W. L., Aufderheide, A. C., Buikstra, J. & Holcomb, T. A. (1994). Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. *Proc Natl Acad Sci U S A* **91**, 2091–2094.

Scott, S. & Duncan, C. (2001). *Biology of Plagues: Evidence from Historical Populations.* Cambridge: Cambridge University Press.

Shapiro, B., Sibthorpe, D., Rambaut, A., Austin, J., Wragg, G. M., Bininda-Emonds, O. R. P., Lee, P. L. M. & Cooper, A. (2002). Flight of the dodo. *Science* **295**, 1683.

Spigelman, M., Matheson, C., Lev, G., Greenblatt, C. & Donoghue, H. (2002). Confirmation of the presence of *Mycobacterium tuberculosis* complex-specific DNA in three archaeological specimens. *Int J Osteoarch* **12**, 393–401.

Stephens, J. C., Reich, D. E., Goldstein, D. B. & 36 other authors, (1998). Dating the origin of the CCR5-Delta32 AIDS-resistance allele by the coalescence of haplotypes. *Am J Hum Genet* **62**, 1507–1515.

Taubenberger, J. K., Reid, A. H., Krafft, A. E., Bijwaard, K. E. & Fanning, T. G. (1997). Initial genetic characterization of the 1918 ''Spanish'' influenza virus. *Science* **275**, 1793–1796.

Taylor, G., Crossey, M., Saldanha, J. & Waldron, T. (1996). DNA from *Mycobacterium tuberculosis* identified in medieval human skeletal remains using PCR. *J Archaeol Sci* **23**, 789–799.

Titball, R. W. & Williamson, E. D. (2001). Vaccination against bubonic and pneumonic plague. *Vaccine* **19**, 4175–4184.

Von Kohl, C. (1911). Historiske Meddelelser om Københavns. Copenhagen, 1911–12. *GEC Gad* **3**, 545–614 (in Danish).

Vreeland, R. H., Rozenwieg, W. D. & Powers, D. W. (2000). Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature* **407**, 897–900.

Waldron, H. (2001). Are plague pits of particular use to palaeoepidemiologists? *Int J Epidemiol* **30**, 104–108.

Willerslev, E., Hansen, A. J., Christensen, B., Steffensen, J. P. & Arctander, A. (1999). Diversity of Holocene life forms in fossil glacier ice. *Proc Natl Acad Sci U S A* **96**, 8017–8021.

WHO. (1999). *Plague Manual: Epidemiology, Distribution, Surveillance and Control* (http://www.who.int/csr/resources/publications/plague/WHO_CDS_CSR_EDC_99_2_EN/en). Geneva: World Health Organization.

Yersin, A. (1894). La peste bubonique à Hong Kong. *Ann Inst Pasteur (Paris)* **8**, 662–667.

Zhang, L., Cui, A., Schmitt, K., Hubert, R., Navidi, W. & Amheim, N. (1992). Whole genome amplification from a single cell: implications for genetic analysis. *Proc Natl Acad Sci U S A* **89**, 5847–5851.

Zink, A., Haas, C. J., Reischl, U., Szeimies, U. & Nerlich, A. G. (2001). Molecular analysis of skeletal tuberculosis in an ancient Egyptian population. *J Med Microbiol* **50**, 355–366.