First report of *Mycobacterium bovis* DNA in human remains from the Iron Age

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Tuberculosis has plagued humankind since prehistoric times, as is evident from characteristic lesions on human skeletons dating back to the Neolithic period. The disease in man is due predominantly to infection with either *Mycobacterium tuberculosis* or *Mycobacterium bovis*, both members of the *M. tuberculosis* (MTB) complex. A number of studies have shown that when conditions permit, surviving mycobacterial DNA may be amplified from bone by PCR. Such ancient DNA (aDNA) analyses are subject to stringent tests of authenticity and, when feasible, are invariably limited by DNA fragmentation. Using PCRs based on single-nucleotide polymorphic loci and regions of difference (RDs) in the MTB complex, a study was made of five Iron Age individuals with spinal lesions recovered from the cemetery of Aymarlyg, South Siberia. A sensitive screening PCR for MTB complex mycobacteria was positive in four out of the five cases. Genotyping evidence indicated that all four cases were due to infection with *M. bovis* rather than *M. tuberculosis* and the data were consistent with the proposed phylogenetic model of the MTB complex. This is believed to be the first report of *M. bovis* causing Pott's disease in archaeological human remains. The study shows that genotyping of ancestral strains of MTB complex mycobacteria from contexts of known date provides information which allows the phylogeny of the model to be tested. Moreover, it shows that loss of DNA from RD4, which defines classic *M. bovis*, had already occurred from the genome over 2000 years before the present.

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INTRODUCTION

Tuberculosis is a chronic infectious pulmonary disease which is caused in humans primarily by *Mycobacterium tuberculosis* and more rarely by *Mycobacterium bovis*. *M. tuberculosis* is transmitted predominantly from human to human via droplet respiratory infection when an infected individual coughs or sneezes (Roberts & Buikstra, 2003). The human-to-human transmission of tuberculosis is reliant on the existence of groups of individuals living in close proximity to one another.

Abbreviations: aDNA, ancient DNA; AMS, accelerator mass spectroscopy; BP, before the present; DR, direct repeat; MTB complex, *Mycobacterium tuberculosis* complex; RD, region of difference.

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The bovine form of tuberculosis is transmitted from animals (such as cattle) to humans through infected milk or meat and is consequently primarily a disease of the stomach and intestinal tract, although it can cause respiratory disease (Cotter *et al.*, 1996; LoBue *et al.*, 2004; Thompson *et al.*, 1993). In developed countries such as the UK, where control measures and herd testing have been in place since the 1930s, bovine tuberculosis accounts for <1% of human tuberculosis cases and tends to be a disease associated with those in close proximity to livestock, such as farmers, veterinary surgeons and abattoir workers (Gutierrez *et al.*, 1997; Robinson *et al.*, 1988; Smith *et al.*, 2004). As such, it is considered to be a spillover infection in humans and is seldom self-maintaining (O'Reilly & Daborn, 1995).

In a small percentage of cases, tuberculosis may affect the skeleton, giving rise to characteristic lytic lesions with minimal new bone formation (Ortner & Putschar, 1985).

Many such cases have been identified in the archaeological record. However, based on morphological examination of osteological lesions, palaeopathologists cannot distinguish between infection due to *M. tuberculosis* or *M. bovis* (Roberts & Buikstra, 2003; Ortner, 1999). Dependent upon DNA survival, polymorphic loci in the genomes of these two organisms permit this distinction to be made, even in ancient cases (Taylor *et al.*, 1999). Whilst a number of groups have now successfully amplified fragmented mycobacterial DNA from archaeological human remains, relatively few have applied genetic typing methods. A recent review of the tuberculosis ancient DNA (aDNA) literature identified the potential for such analyses to contribute to the debate on the evolution of the *M. tuberculosis* (MTB) complex and to the nature of the host–pathogen interaction (Donoghue *et al.*, 2004).

In the present study we examined osteological samples obtained from five individuals with skeletal evidence of tuberculosis retrieved from the cemetery of Aymyrlyg in the Ulug-Khemski region of Tuva, South Siberia. South Siberia is one of the areas of the world with the oldest traditions of pastoralism and it was recognized that there was the potential to identify biomolecular evidence for the bovine form of the disease, which has previously eluded identification (Donoghue *et al.*, 2004). As such, the objectives of the aDNA analysis were to confirm that the lesions were due to tuberculosis and to determine whether the individuals had been infected with either *M. bovis* or *M. tuberculosis*.

METHODS

Radiocarbon dating. Radiocarbon dating using accelerator mass spectroscopy (AMS, Table 1) yielded dates ranging from approximately 1761 to 2199 years BP, placing the remains within the Iron Age period of South Siberia. This dates burials XXXI.34 and XXXI.77 to

the transition period between the Uyuk (7th–3rd centuries BC) and Shurmak (2nd century BC–5th century AD) Cultures and the other three burials within the later Shurmak or Synnchyurek Culture.

DNA extraction. Bone fragments ranging from 70 to 420 mg in weight were removed from within the body of the vertebrae and the rib using sterile probes and scalpel blades. All fragments were crushed to a powder in sterile pestles and mortars. The NucliSens isolation kit from bioMérieux was used to prepare DNA extracts as previously described (Taylor *et al.*, 2005).

PCR. Hot-staRT-PCR was performed in a final volume of 25 µl using the Excite Core kit from BioGene with minor modification to the manufacturer’s instructions. Additional *Taq* polymerase (0.4 µl, 2 U) was added to the master mix to allow for PCR inhibitors in bone extracts. After an initial denaturation step (8 min at 95 °C), 45 cycles of amplification were performed as follows: denaturation at 95 °C for 10 s, annealing at 58 to 62 °C for 30 s, extension at 72 °C for 20 s. SYBR Green was included in the PCR master mixes at a final dilution of 1/55 000, and reactions were performed and monitored with a Corbett RotorGene 3000 real-time PCR platform. Melting analysis was performed with the RotorGene software and all products were also run on 3 % agarose checker gels. Cycle sequencing of PCR products was performed as previously reported (Taylor *et al.*, 2003).

Extracts from the Aymyrlyg burials were screened using two methods which amplify repetitive elements specific to the MTB complex, *IS6110* and *IS1081* (Dziadek *et al.*, 2001). Multi-copy genes and repetitive elements such as these make good targets when attempting to demonstrate the presence of pathogen DNA in the archaeological record (Cano, 1996). *IS6110* is multi-copy in the majority of extant strains of *M. tuberculosis* (Plikaytis *et al.*, 1993). Most isolates of the MTB complex contain six copies of the insertion sequence *IS1081* (Collins & Stephens, 1991), so this element is therefore theoretically a better target when testing for *M. tuberculosis* isolates, which have few copies or even lack *IS6110* (Yuen *et al.*, 1993), and *M. bovis*, which usually contains a single *IS6110* copy in the direct repeat (DR) region of the genome (Hermans *et al.*, 1991). However, variation in *IS6110* copy number has been associated with *M. bovis* isolates of different geographical origin (Liebana *et al.*, 1997).

Table 1. Details of burials and sample sites

Age-at-death and sex determinations were made on the basis of morphological characteristics (Buikstra & Ubelaker, 1994; Ferembach *et al.*, 1980). F, female; M, male. AMS dates obtained from the individuals were calibrated using OxCal v3.5 (Bronk-Ramsey, 2000; Stuiver *et al.*, 1998).

Context	Age (years)	Sex	Nature of lesions	Sample sites	Likelihood of TB, based on osteology	AMS calibrated date
XXXI.34	25–35	F	Lytic lesions in eight vertebrae	i. 3rd lumbar vert. ii. 4th lumbar vert.	Probable	360–170 BC (95.4 %)
XXXI.77	25–35	M	Lytic lesions in a total of nine vertebrae; lytic lesions in left knee joint	i. 5th lumbar vert. ii. 6th lumbar vert.	Probable	360–170 BC (95.4 %)
XXXI.85 Sk. 1	25–35	F	New bone formation, lumbar vertebrae	i. 4th lumbar vert. ii. 5th lumbar vert.	Possible	20–140 AD (95.4 %)
XXXI.101	7.5–8.5	?	Lytic lesions in all vertebrae; new bone formation, entire skeleton	i. Lumbar vert. ii. Rib fragment	Probable	210–390 AD (92.4 %)
XXXI.163	35–45	F	New bone formation, lumbar vertebrae	i. 4th lumbar vert. ii. 5th lumbar vert.	Possible	70–230 AD (95.4 %)

Polymorphic loci and regions of difference. The extracts were also tested using a series of genotyping PCRs to place any positive cases within the evolutionary scenario for the MTB complex (Brosch *et al.*, 2002). This scheme has been compiled using comparative genomics of extant strains of members of the MTB complex (*Mycobacterium canettii*, *M. tuberculosis*, *M. africanum*, *M. microti*, *M. bovis* and *M. bovis* BCG). The assays included analysis of polymorphic loci within the *oxyR* pseudogene (*oxyR285*) and the pyrazinamidase gene (*pncA159*), which can be used to distinguish between *M. tuberculosis* and *M. bovis* (Scorpio *et al.*, 1997; Sreevatsan *et al.*, 1996). PCR primers flanking deletions within the MTB complex were also used to assess the status of key regions of difference (RD). The deletions tested for were RD12, RD13, RD4 and RD17 and TbD1. This last deletion has been used to distinguish 'ancestral' from 'modern' isolates of TB, as the region is retained only in *M. bovis* and in a subgroup of the ancestral group 1 isolates of *M. tuberculosis* (Brosch *et al.*, 2002; Sreevatsan *et al.*, 1997). However, it may be more appropriate to associate the presence or absence of the TbD1 locus with geographical areas or distinct lineages, as the population genetics of *M. tuberculosis* is now seen as being geographically related (Gagneux *et al.*, 2006). Primer sequences for novel RD methods are shown in Table 2. Details of the TbD1, the *oxyR285* and the *pncA169* methods have been previously reported (Taylor *et al.*, 2005).

Measures to exclude modern contamination. Measures to prevent contamination of extracts with previously generated amplicons were applied throughout the study. These have been described in detail previously (Mays *et al.*, 2001; Taylor *et al.*, 2006). The focus was on physical barriers with separate areas for extraction, PCR set-up and analysis. Surfaces and equipment (centrifuges, rotors, pipettes, etc.) were cleaned before each experiment. Filter tips were used routinely. Control extractions were used to monitor the efficacy of these procedures. Template (water) blanks were alternated with samples in the PCR machines to screen for contamination. Positive controls were either excluded from amplifications or were run in separate laboratories to minimize the opportunities for contaminating aDNA extracts.

Reproducibility

Imperial College, London. Experiments were undertaken by two of us (R.H. and G.M.T.) separated by a period of 3 months to assess the reproducibility of any positive findings. R.H. assayed extracts

with IS6110, IS1081 and *oxyR285* methods, G.M.T. with these loci plus *pncA169*, all RD PCRs and TbD1 primers. Separate bone extracts were used for these two series of experiments.

Second-centre confirmation. Author P.R. analysed fresh extracts at a second laboratory, in this case the Department of Genetics at the Institute of Child Health, University College London. No research on tuberculosis had previously been conducted within this department. The experiments were limited to confirmation of the MTB complex by IS1081 PCR and *oxyR285* genotyping to determine species. All primers and PCR reagents for this phase of the study were newly synthesized or purchased separately.

RESULTS

A total of five skeletons were the subject of the present biomolecular study (Table 1). Three of the skeletons displayed infective lesions that are considered to be indicative of tuberculosis. In addition, two individuals displayed new bone formation on the anterior surfaces of one or more of their vertebrae. It was considered possible that these lesions were due to some form of gastrointestinal infection, which could have been tuberculosis (Table 1). All of the skeletons were Iron Age in date (Table 1) and came from a society that is believed to have practised a semi-nomadic pastoralist form of economy. This would have taken the form of a pattern of repeated seasonal migrations or 'shifts' with herd animals within a well-defined territory. The chief herding animals of peoples from this culture were sheep, goats and cattle. Horses were also kept and would have been used for transport, as would cattle and yaks (Vainshtein, 1980).

Mycobacterial DNA was successfully amplified and genotyped from four of the five individuals with skeletal lesions. The PCR results for the screening and genotyping PCRs are summarized in Table 3. Extracts ($n=8$) prepared from four of the burials were found to be IS1081 positive, a sensitive marker for the MTB complex. Genotyping PCRs were

Table 2. Details of novel PCR methods used in the present study

PCR	Primer	Sequence (5'–3')	[Mg ²⁺] (mM)	Anneal. temp.	Amplicon size (bp)
IS1081	F2	CTGCTCTCGACGTTTCATCGCCG	1.5	58	135
	R2	GGCACGGGTGTCGAAATCACG			
RD12 flanking	Forward	CACACCTGGTTCGTGTTGCG	2.0	58	123
	Reverse	GGTGAAGAACGTCGTCAAGCACAT			
RD13 flanking	Forward	ATCGCTCGTTCGTCTGGCTTC	2.0	60	136
	Reverse	GGCAAGACCGGGCCTTTGAC			
RD4 flanking	F2	TGTGAATTCATACAAGCCGTAGTC	2.0	58	142
	R1	CCCGTAGCGTTACTGAGAAATTGC			
RD17 flanking	Forward	CGTGGGTGGACAGTGTCTCATGGAC	2.0	62	118
	Reverse	GCCCCGCTGTGCACGACCGCCA			
RD17 internal	Forward	GTGATCGAGAAGAAACGGCGGTAGC	2.0	62	96
	Reverse	GGACGGCACCGGCCCGCTGT			

applied to the more strongly positive extracts from each of the four positive cases. Amplification and sequencing of *oxyR285* from all four cases showed an adenine (A) base at this locus, indicative of an *M. bovis* isolate (cf. G in *M. tuberculosis*). These findings were replicated independently by both workers at Imperial College and by author P. R. at the second centre in University College London.

The *pncA169* locus was amplified from two cases, XXXI.85 Sk. 1 and XXXI.163, and found to be guanine (G), again consistent with *M. bovis* strains (cf. C in *M. tuberculosis*). The IS6110 PCR, normally a very sensitive marker of remnant tuberculosis DNA, was found to be positive in only three out of eight of the same IS1081-positive extracts. Two of these were from the same burial, XXXI.163, while the other positive result was derived from skeleton XXXI.77 (Table 3). Extraction and template blanks were consistently negative, showing that measures to avoid contamination were effective.

Skeleton XXXI.101, a 7.5–8.5-year-old child, was always negative for IS1081 and IS6110 and so other less sensitive PCRs were not applied to this case. However, this individual displayed Pott’s disease of the spine and other characteristic lesions of tuberculosis, and it is considered likely that the negative result may have been due to the poorer preservation of the immature bone relative to the adult remains.

PCR performed with primers flanking the RD12, RD13 and RD4 deletions yielded products of the expected size, showing that these deletion events had occurred in the strains recovered from the Aymyrylg burials. The status of the RD17 deletion was studied in two cases, burials XXXI.77 and XXXI.85. Use of flanking primers for RD17 failed to produce a specific amplicon but internal primers generated products of the expected size (96 bp), in both cases. The identity of the PCR product was confirmed in burial XXXI.77 by sequencing.

Extracts prepared from burials XXXI.63 and XXXI.77 were generally more robust in terms of PCR products obtained using the RD single-copy PCR methods, suggesting that

template integrity was better in these cases. All the positive tests indicated that four out of five of the Siberian individuals were infected with bovine tuberculosis. The fact that *M. bovis* was the species involved may explain the relatively poor IS6110 data, as this marker is single copy in the majority of extant *M. bovis* strains, and this may also be the case in these older isolates.

DISCUSSION

The MTB complex group of organisms, comprising *M. canetti*, *M. tuberculosis*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. caprae*, classic *M. bovis* and *M. bovis* BCG, are believed to have developed from a diverse group of smooth tubercle bacilli closely related to *M. canetti*. Genetic variation in extant strains of *M. canetti* and other smooth tubercle bacilli from East Africa suggests that the last common ancestor of these related but diverse groups dates back to almost 3 million years BP (Gutierrez *et al.*, 2005). In contrast, limited synonymous nucleotide diversity in present-day MTB complex mycobacteria suggests that this detachment from the progenitor group and subsequent clonal expansion has taken place within a narrower time-frame, estimated at up to 35 000 years BP (Gutierrez *et al.*, 2005; Sreevatsan *et al.*, 1997).

In recent years, bioarchaeological studies have begun to examine cases of tuberculosis from the archaeological record and to obtain genotyping data. Evidence for *M. tuberculosis* as the causative species has been obtained from mediaeval sites in Europe (Taylor *et al.*, 1999; Mays *et al.*, 2001; Fletcher *et al.*, 2003), from Iron Age Britain (Taylor *et al.*, 2005) and from Middle Kingdom Egypt (Zink *et al.*, 2003). Limited evidence from spoligotyping of the Egyptian cases has shown an absence of spacers 33–36 in the DR region of the genome, suggesting strains belonging to phylogenetic group 2 in the Musser model of the MTB complex (Sreevatsan *et al.*, 1997). Isolates of *M. tuberculosis* from this group have lost the TbD1 region, and this work indicates that this deletion may have occurred at least 2500 years BP. Loss of TbD1 is specific to *M. tuberculosis* strains and is a marker which has

Table 3. Summary of PCR data

+, PCR positive; –, PCR negative. A, adenine; G, guanine; ND, not determined.

Burial no. [sample]	IS6110	IS1081	oxyR285	pncA169	RD12 flanking	RD13 flanking	RD4 flanking	RD17 internal	TbD1 internal
XXXI.163 [ii]	+	+*†	+ (A)*†	+ (G)*	+	+	+	ND	+
XXXI.77 [i]	+	+*†	+ (A)*†	+	+	+	+	+	+
XXXI.85, Sk. 1 [i]	–	+	+ (A)*	+ (G)*	+	–	+	+	–
XXXI.34 [i]	–	+	+ (A)*†	ND	–	–	+	ND	–
XXXI.101 [i] and [ii]	–	–	ND	ND	ND	ND	ND	ND	ND

*Identity of PCR product confirmed by direct DNA sequencing, laboratory 1.
†PCR also performed and confirmed by direct sequencing at the second laboratory.

been used to distinguish 'modern' strains from those which are more closely related to the common ancestor of the complex and those from certain geographical origins (Brosch *et al.*, 2002; Sun *et al.*, 2004). Consistent with the antiquity of this event implied from the spoligotyping of Egyptian remains, we have recently reported that the TbD1 region had been deleted in a strain from an Iron Age burial with Pott's disease from Dorset, UK (Taylor *et al.*, 2005). 'Modern' strains may be older than the term suggests. Zink *et al.* (2003) also observed spoligotype fingerprints consistent with *M. africanum* in older Egyptian burials but found no evidence for bovine tuberculosis.

The present study is believed to be the first to document bovine tuberculosis in human remains and shows that the RD status and polymorphisms in these Iron Age cases are in agreement with the evolutionary scheme proposed by Brosch *et al.* (2002). Tests for RDs 12, 13 and 4 showed that these loci had all been deleted from the Siberian cases whereas the TbD1 and RD17 regions were still present. These findings, together with characteristic nucleotide polymorphisms in *pncA169* and *oxyR285*, are typical of classic *M. bovis*, so it is probable that these isolates were derived from strains which had originally adapted to cattle. Our findings indicate that the RD4 deletion associated with *M. bovis* had already been lost from the genome at least 2000 years BP. For the most part, little is known of the absolute chronology of the deletions in the bovine lineage, although loss of five regions occurring over a 50 year period of intensive passage in laboratories worldwide has been documented in BCG vaccine strains (Behr *et al.*, 1999).

Based on deletion subset patterns in *M. bovis* isolates, there are three lineages (a–c) in which the RD17 region is present and one (d) in which it has been lost (Mostowy *et al.*, 2005). The majority of UK bovine spoligotypes, including the sequenced strain AF 2122/97, fall into this latter group, having lost RD17. The region is retained in two spoligotypes, GB35 and GB54, both relatively rare causes of bovine tuberculosis in the UK but more frequently reported in France (Haddad *et al.*, 2001). The observation that the strain of *M. bovis* infecting burials XXX1.77 and XXX1.85 retained RD17 therefore distinguishes them from strains commonly isolated in the UK. Further sampling would be necessary to identify from which of the three lineages with RD17 this strain was derived.

As bovine tuberculosis is not considered to be self-maintaining in man (O'Reilly & Daborn, 1995) we infer that disease in these human remains, spanning several centuries, reflects continued exposure of the population to an infected animal reservoir host or hosts. During the Bronze Age, the mountain-steppe tribes of Tuva probably kept more cattle than sheep or goats (Gryaznov, 1969). By the Iron Age, sheep and goats almost certainly accounted for more than half of all livestock (Vainshtein, 1980). Therefore, it is interesting to note that the disease was not caused by the caprine-adapted variant of *M. bovis* ('*M. caprae*') first identified in Spanish goats. This is characterized by

retention of the RD4 region and the *pncA169C* mutation usually associated with *M. tuberculosis*. Although first isolated from goats, this member of the *M. bovis* lineage can also cause disease in cattle (Aranaz *et al.*, 2003). Next in frequency after sheep and goats would have come horses and cattle. Cattle husbandry continued as an important part of the economy for milk, meat and traction. During harsh winters, cows' milk would have been fed to young lambs and kids when grazing was difficult (Vainshtein, 1980), so these animals, as well as cattle, may have acted as reservoirs of disease. It is recognized that classic *M. bovis* has a wide host range and can cause disease in sheep and goats, although in some countries, such as Britain, this is a relatively rare event (Malone *et al.*, 2003). It would therefore be interesting to study the faunal remains from Amyrlyg for *M. bovis* aDNA, but this poses a number of problems due to the disarticulated, fragmentary and often scavenged nature of faunal remains at archaeological sites. This is compounded by the degree to which the skeleton may be affected in different species, the age at slaughter and the lack of diagnostic criteria for skeletal evidence of tuberculosis in animals (Mays, 2005).

DNA from archaeological contexts is invariably degraded, and sampling is destructive and usually regulated; hence the biomolecular evidence which can be recovered from human remains is very limited. However, several lines of evidence have shown *M. bovis* DNA in skeletal remains from these semi-nomadic Iron Age pastoralists. We speculate that this resulted from their continued close proximity to infected cattle or to another species which acted as either a spillover or reservoir host of the disease. Consumption of raw milk and poorly cooked meat are the most probable routes of infection. This was certainly the situation for developed Western countries in the era before various control measures, such as herd testing and pasteurization of milk, were introduced (O'Reilly & Daborn, 1995).

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