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Complications in the study of ancient tuberculosis: Presence of environmental bacteria in human archaeological remains

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ABSTRACT

There are many reports of ancient DNA from bacteria of the *Mycobacterium tuberculosis* complex (MTBC) being present in skeletons with and without osteological indications of tuberculosis. A possible complication in these studies is that extracts might also contain DNA from the microbiome of the individual whose remains are being analysed and/or from environmental bacteria that have colonised the skeleton after death. These contaminants might include 'mycobacteria other than tuberculosis' (MOTT), which are common in the environment, but which are not normally associated with clinical cases of tuberculosis. In this paper we show that MOTT of various types, as well as bacteria of related genera, are present in most if not all archaeological remains. Our results emphasise the complications inherent in the biomolecular study of archaeological human tuberculosis. The specificity of any polymerase chain reaction directed at the MTBC cannot be assumed and, to confirm that an amplification is authentic, a sequencing strategy must be applied that allows characterisation of the PCR product. Any variations from the reference MTBC sequence must then be checked against sequence data for MOTT and other species to ensure that the product does actually derive from MTBC. Our results also illustrate the challenges faced when assembling MTBC genome sequences from ancient DNA samples, as misidentification of MOTT sequence reads as MTBC would lead to errors in the assembly. Identifying such errors would be particularly difficult, if not impossible, if the MOTT DNA content is greater than that of the authentic MTBC. The difficulty in identifying and excluding MOTT sequences is exacerbated by the fact that many MOTT are still uncharacterized and hence their sequence features are unknown.

1. Introduction

Tuberculosis (TB) is caused by members of the *Mycobacterium tuberculosis* complex (MTBC) of bacteria. These species include *M. tuberculosis*, which is the most frequent cause of human TB, the less common human pathogens *Mycobacterium africanum* and *Mycobacterium canettii*, as well as *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium pinnipedii* and *Mycobacterium caprae*, which are primarily responsible for TB in animals but have also been known to infect humans (Gutiérrez et al., 1997; Van Soolingen et al., 1997, 1998; Aranaz et al., 2003; Kiers et al., 2008). Although principally a pulmonary disease, 3–5% of modern-day untreated TB patients develop bone changes following spread of bacteria through the body via the blood and lymphatic systems (Jaffe, 1972). Typical lesions include collapse of the lower thoracic and upper lumbar vertebrae resulting in a curvature of the spine, called Pott's disease, and damage to major joints, particularly the hip or knee (Resnick, 2002). More non-specific bone changes have also been identified as potentially related to TB in archaeological skeletons, such as granular impressions and new bone formation on the endocranial surface of the skull, new bone formation on the visceral surfaces of the ribs, hypertrophic pulmonary osteoarthropathy and dactylitis (see Roberts and Buikstra, 2003:99–109 for a summary). These non-specific lesions can be recognized in archaeological skeletons, and although not specific for TB provide indications of the possible prevalence of the disease in past societies.

The excavation of skeletons with indications of TB, and the likely presence of MTBC bacteria in the bones at time of death, has made TB an attractive target for ancient DNA (aDNA) studies. The first report of *M. tuberculosis* aDNA detection (Spigelman and Lemma,

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1993) has been followed by a substantial number of publications describing the use of the polymerase chain reaction (PCR) to detect MTBC in human bones and teeth (e.g. Baron et al., 1996; Taylor et al., 1996, 1999; Faerman et al., 1997; Haas et al., 2000; Mays et al., 2001, 2002; Zink et al., 2001, 2003, 2005; Mays and Taylor, 2003; Donoghue et al., 2005; Müller et al., 2014a, 2014b), mummified soft tissue (e.g. Salo et al., 1994; Nerlich et al., 1997) and calcified pleura (e.g. Donoghue et al., 1998), spanning a time frame from as early as 9000 BC (Hershkovitz et al., 2008) through the Iron Age (e.g. Mays and Taylor, 2003) and the Medieval periods (e.g. Faerman et al., 1997) and up to modern times (e.g. Zink et al., 2005). More recently, next generation sequencing (NGS) methods, with or without pre-capture of MTBC aDNA, have been used to obtain genomic data from skeletal and mummified human remains (Bouwman et al., 2012; Chan et al., 2013; Bos et al., 2014).

A possible complication in the study of samples for MTBC aDNA is the presence of DNA from the microbiome of the individual whose remains are being analysed and/or from environmental bacteria that have colonised the skeleton after death (Wilbur et al., 2009; Tsangaras and Greenwood, 2012; Müller et al., 2015). These contaminants might include 'mycobacteria other than tuberculosis' (MOTT), which are common in the environment and may occasionally cause opportunistic disease, but which are not normally associated with clinical cases of TB. The genus *Mycobacterium* is often classified into two major groups, slowly and rapidly growing mycobacteria, a phenotypical division which appears to be supported by genomic data (e.g. Khan and Yadav, 2004; Kweon et al., 2015; Wang et al., 2015). The former group comprises the MTBC and other opportunistic pathogens (human or animal), whereas fast-growing mycobateria are predominantly non-pathogenic (Wang et al., 2015). The PCRs used to detect MTBC are generally considered to be specific when used with clinical samples, but most clinical samples do not contain extensive MOTT contamination. False-positives might therefore occur when they are used with more complex extracts, such as those from skeletons that have been buried in the ground for centuries (Wilbur et al., 2009). We have recently shown that this is the case with the standard PCR used to detect the IS6110 sequence, which was previously thought to give positive results only with DNA from the MTBC, but which also amplifies fragments of the same length from environmental bacteria that are present in at least some human archaeological skeletons (Müller et al., 2015). Contamination with MOTT species might also complicate assembly of MTBC genome sequences after NGS of archaeological bone or dental samples, if sequence reads derived from environmental species are mistaken for ones representing an MTBC member (Bouwman et al., 2012).

In this paper we assess the extent and nature of the MOTT and broader bacterial content of human archaeological skeletons, including several displaying potential osteological indicators of TB. We present the results of two independent analyses. First, we report the identities of MOTT present in different skeletons and show the taxonomic relationships of these with MTBC. Second, we provide examples of the non-specificity of PCRs directed at the MTBC, and describe the contaminating sequences and their likely derivation.

2. Materials and methods

2.1. Samples

We studied 22 samples taken from bones or teeth from 21 skeletons from 1st–19th centuries AD from 13 sites in Britain and three from continental Europe (Table 1). Fourteen of these skeletons displayed pathological alterations possibly suggesting infection with MTBC. In previous work (Müller et al., 2014a) we have obtained reproducible IS6110 PCR products from 11 of these samples, checked by sequencing of the cloned amplicons, and less reproducible results for IS6110 and/or a second insertion element, IS1081 with seven other samples. The remaining four samples gave no evidence for the presence of MTBC aDNA (Table 1). Most of the samples were from individuals buried in earth-cut graves, with direct exposure to the surrounding soil, but three individuals were recovered from vaults (St George's Crypt 4006, St George's Crypt 5003, St Peter's Collegiate Church 62).

Precautions used to prevent contamination of samples with exogenous DNA, and the methods used to prepare DNA extracts, are described in Müller et al. (2015).

2.2. Polymerase chain reactions

Polymerase chain reactions were set up in 30 μl reactions containing 2.5–5.0 μl DNA extract, 1 × AmpliTaq Gold PCR Master Mix (Applied Biosystems), 400 nM each primer, 1% BSA and ultrapure water. PCRs were run for one cycle for 7 min at 95 °C, followed by 40–45 cycles each consisting of 1 min at the annealing temperature, 1 min at 72 °C and 1 min at 95 °C, with a final extension for 10 min at 72 °C. Six genetic loci were studied (Table 2) using the primer sequences and PCR conditions given in Table 3. Amplification products were run on a 2% agarose gel and purified products cloned into *Escherichia coli* XL1-Blue competent cells (Agilent) using the CloneJet PCR cloning kit (Thermo Scientific). Clones were sequenced (GATC Biotech, Cologne) and aligned with the respective reference sequences using Geneious version 7.1.7 (available from http://www.geneious.com/). BLAST (Altschul et al., 1990) was used to compare sequences with the GenBank nucleotide database. For hsp65, 187 bp fragments obtained after primer removal were also aligned to the homologous regions of 168 mycobacteria from the 'List of Prokaryotic names with Standing in Nomenclature' (Euzéby, 1997). A maximum likelihood (ML) tree was created using Mega version 6 (Tamura et al., 2013). The Jukes-Cantor model for nucleotide substitution (Jukes and Cantor, 1969) was applied and 1000 bootstrap replicates were used to determine the robustness of the tree topology. The tree was visualized with FigTree version 1.4.2 (available from http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

3.1. PCRs directed at the mycobacterial heat-shock protein gene hsp65

We applied a genus-specific PCR directed at the heat shock protein gene hsp65 (Khan and Yadav, 2004) to ten samples, cloned the PCR products, and sequenced 2–6 clones per sample in order to identify the mycobacterial species that were present. None of the sequences exactly matched any of the mycobacterial hsp65 entries contained in GenBank, but BLAST searches identified all sequences as most likely deriving from MOTT species. Only one sequence type was obtained for each of the individuals from Ashchurch 705, Horncastle 20, Kempston 3902 and Obeliai 143A, but all the other samples displayed at least two different sequence types (Supplementary Fig. 1).

A maximum likelihood tree constructed from the clone sequences and the equivalent hsp65 regions of 168 mycobacterial species displayed the expected division of *Mycobacterium* into slow and rapid growers, with the exception of a few species which did not fall into their respective clusters (Fig. 1). The tree suggests that the sequences obtained from individuals Ashchurch 705 and Horncastle 20 fall within the group of slow-growing mycobacteria, and those from Kempston 3902, Obeliai 143 and Queensford Mill

Table 1
Previous aDNA results for the samples that were studied (Müller et al., 2014a, 2014b).

Sample	Date	Bones showing possible TB lesions	MTBC aDNA status[a]	MTBC strain identification
Ashchurch Bridge 705	129–317 calAD	Ribs	A (rib), B (tooth)	Yes
Ashton 118	257–415 calAD	None	B	No
Auldhame 43	1280–1394 calAD	Ribs	A	Yes
Easington/Ganstead 25183	2nd AD	Vertebrae	D	No
Horncastle 6	136–335 calAD	None	D	No
Horncastle 7	3rd AD	None	D	No
Horncastle 20	3rd AD	None	B	No
Kempston 3902	3rd-4th AD	None	C	No
Obeliai 143A	5th–6th AD	Vertebrae	B	No
Queensford Mill 157	236–382 calAD	Vertebrae	C	No
Saint Amé 20	16–18th AD	Ribs	A	Yes
Shchekavitsa 8	late 10th–12th AD	Endocranium	A	Yes
St George's Crypt 4006	mid-19th AD	Ribs, mandible, calcaneus	A (rib), B (tooth)	Yes
St George's Crypt 5003	mid-19th AD	None	A	Yes
St Peter's Church 1390	1016–1155 calAD	Ribs	A	Yes
St Peter's Collegiate Church 28	19th AD	Ribs	A	Yes
St Peter's Collegiate Church 62	19th AD	Ribs, humeri, scapulae, radius	A	Yes
Weston-super-Mare 01	Roman	Vertebrae, humerus	D	No
Wheatpieces 4	28–211 calAD	None	B	No
Whitefriars 657	18th–19th AD	Ribs	A (tooth), C (rib)	Yes (tooth), No (rib)
Whitefriars 10,466	18th–19th AD	Ribs, endocranium	A	Yes

[a] A, previously identified to definitely contain MTBC aDNA; B, previously identified to probably contain MTBC aDNA; C, previously identified to possibly contain MTBC aDNA (Müller et al., 2014a).

Table 2
Genetic loci studied in each sample.

Sample	Sampled element	hsp65	qcrB	rpoB^{3243}	rpoB^{2646}	gyrA^{285}	katG^{1388}
Ashchurch Bridge 705	Rib	✓	✓	✓			
Ashton 118	Femur				✓		
Auldhame 43	Rib			✓			
Easington/Ganstead 25183	Vertebra	✓					
Horncastle 6	Tibia	✓					
Horncastle 7	Long bone	✓					
Horncastle 20	Radius	✓					
Kempston 3902	Femur	✓		✓	✓		
Obeliai 143A	Tibia	✓					
Queensford Mill 157	Femur	✓					
Saint Amé 20	Rib		✓	✓		✓	
Shchekavitsa 8	Femur					✓	
St George's Crypt 4006	Rib	✓		✓			
St George's Crypt 5003	Rib					✓	
St Peter's Church 1390	Rib		✓	✓			
St Peter's Collegiate Church 28	Rib		✓		✓		
St Peter's Collegiate Church 62	Rib		✓	✓		✓	
Weston-super-Mare 01	Vertebra	✓					
Wheatpieces 4	Femur				✓		
Whitefriars 657	Rib, tooth		✓			✓	✓ (rib)
Whitefriars 10466	Rib		✓	✓			

Table 3
Details of polymerase chain reactions.

Locus	Primers (5′ → 3′)[a]	Annealing temperature (°C)	Number of cycles	Amplicon size (bp)
hsp65	F: CTGGTCAAGGAAGGTCTGCG R:GATGACACCCTCGTTGCCAAC	62	40	228
gyrA^{284}	F: CCGGTCGGTTGCCGAGACCA R: GCGGGTAGCGCAGCGACCAG	70	45	104
katG^{1388}	F: CGAAGCCGAGATTGCCAGCCT R:CGCTACCACGGAACGACGACG	70	45	107
qcrB	F: GCTCGCAGCCAGACTTCTACAT R: GGGCGGGAATGGTGTGA	64	45	96
rpoB^{2646}	F: TGCGTGTGTATGTGGCTCAGAAAC R: AAGGAACGGCATGTCCTCAACC	67	45	116
rpob^{3243}	F: GCCAGCGGTTCGGGGA R: TCGGACTTGATGGTCAACAGC	66	45	88

[a] F, forward primer; R, reverse primer.

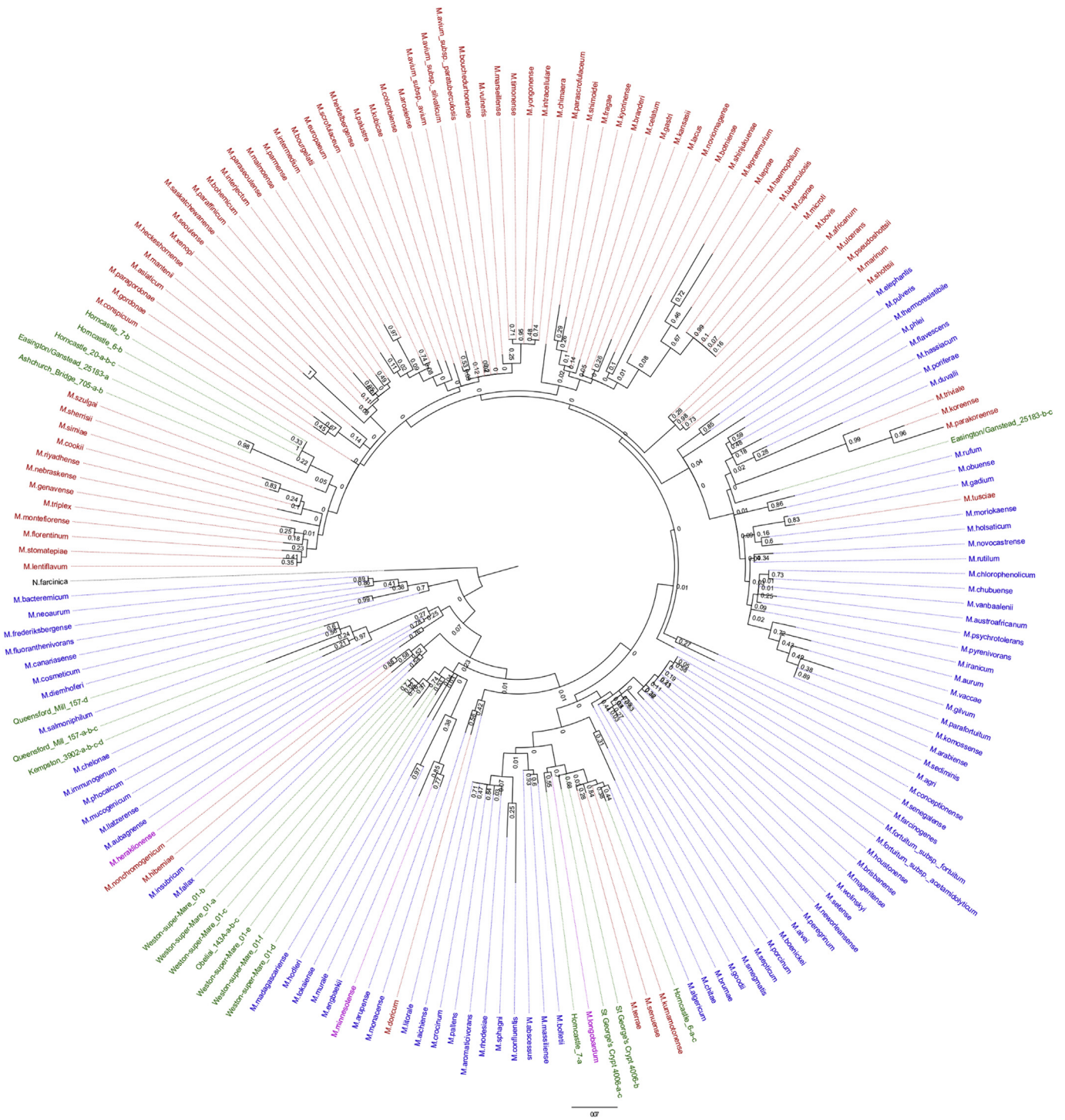

Fig. 1. Phylogenetic tree comparing the hsp65 sequences obtained from samples analysed in this study (green) to 168 extant mycobacteria (Supplementary Dataset). Slow-growing mycobacteria are in red, fast-growing in blue and intermediate ones in pink. The phylogenetic tree was constructed by the maximum likelihood method based on the Jukes-Cantor nucleotide substitution model (Jukes and Cantor, 1969). Initial tree(s) for the heuristic search were obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013) and visualized with FigTree version 1.4.2 (available from http://tree.bio.ed.ac.uk/software/figtree/). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

157 and Weston-super-Mare 01 to fast-growing species. Those from St George's Crypt 4006 fell into a cluster comprising slow, fast and intermediate growing species. Mixtures of slow and fast growing mycobacterial sequences were obtained from Easington/Ganstead 183. Mixed sequence types were also obtained from Horncastle 6 and Horncastle 7, each of these samples giving one sequence that was identical to those obtained from Horncastle 20 and falling into the group of slow growers, together with additional sequences located in the cluster of slow-, fast- and intermediate-growing species.

3.2. PCRs directed at single nucleotide polymorphisms

In previous work we typed single nucleotide polymorphisms (SNPs) in the *M. tuberculosis* genome in order to compare the strains present in different archaeological samples (Müller et al., 2014b). We observed that some PCRs directed at the subunit A of the DNA gyrase (gyrA), catalase-peroxidase (katG), ubiquinol-cytochrome C reductase (qcrB), and two regions within the β subunit of the RNA polymerase (rpoB) sometimes gave products of the expected size but with sequences not corresponding to MTBC. Fifteen samples (Table 2) gave non-specific amplification products for at least one of these five targets (Supplementary Figs. 2–6). For six of these samples (Ashton 118, Kempston 3902, Shchekavits 8, St George's Crypt 5003, Wheatpieces 4, Whitefriars 657 rib) the non-specific products were the only ones obtained. Most amplicon clones of the other nine samples also included the authentic *M. tuberculosis* target, with the exception of some samples yielding only non-specific sequences for rpoB^{3243} (Ashchurch 705, Saint Amé 20, St Peter's Church 1390, Whitefriars 10466).

BLAST analysis (Supplementary Table 1) revealed that, for most of the samples, the closest matches to the non-specific rpoB^{2646} and rpoB^{3243} sequences were the equivalent loci from bacterial genera other than *Mycobacterium*, including *Ilumatobacter*, *Strepromyces*, *Cellvibrio*, *Cellulomonas* and *Micromonospora* for rpoB^{2646} and *Pelotomaculum*, *Acidimicrobium*, *Clavibacter*, *Kineococcus*, *Frankia*, *Ilumatobacter*, *Nakamurella*, *Segniliparus*, *Phycisphaera*, *Streptomyces*, *Leifsonia*, *Thermus*, *Methylobacterium*, *Kribella* and *Dermacoccus* for rpoB^{3243}. In contrast, the sample from individual St George's Crypt 4006 gave rpoB^{3243} sequences identical to MOTT species *Mycobacterium avium*, *Mycobacterium avium* subsp. *paratuberculosis*, *Mycobacterium kansasii*, *Mycobacterium neoaurum*, *Mycobacterium branderi*, *Mycobacterium celatum*, *Mycobacterium kyorinense*, *Mycobacterium mageritense*, *Mycobacterium rhodesiae*, *Mycobacterium smegmatis*, *Mycobacterium vanbaalenii*, *Mycobacterium barrassiae* and a strain of the genus *Nakamurella*, and that from Saint Amé 20 gave rpoB^{3243} sequences with highest similarity to these MOTT species but also members of the genera *Nakamurella* and *Segniliparus*. The sequences of the non-specific gyrA and qcrB amplicons again showed highest similarity with MOTT species, but with members of the MTBC as well as *Rhodococcus*, *Nocardia* and/or *Blastococcus* also giving high scores. The sequence obtained from Whitefriars 657 for katG displayed highest similarity with MTBC.

4. Discussion

Our studies of the hsp65 gene show that MOTT of various types are present in most if not all archaeological remains. Each of the ten samples that we studied gave hsp65 amplicons which, after cloning, yielded sequences not present in Genbank but which clearly derived from mycobacterial species according to their position within an ML tree. In the case of the three Horncastle samples, some of the sequences were identical, which is a clear indication that they come from an environmental MOTT in the burial and/or storage environment, or from the 'mycobacteriome' of the human body, such as the oral cavity and upper respiratory tract which harbours diverse non-tuberculous mycobacterial species even in healthy individuals (Macovei et al., 2015). The same is probably true in those instances where there are only small differences between the sequences obtained from a sample, such as Queensford Mill 157, St George's Crypt 4006 and Weston-super-Mare 01 (Supplementary Fig. 1). Although pseudogenes, with variable sequences compared with the functional version, are present in bacterial genomes (Lerat and Ochman, 2005), the variations seen in these hsp65 amplicons are likely to be due to diagenetic sequence changes and/or PCR error (Gilbert et al., 2003, 2007; Brotherton et al., 2007) rather than variability in the original sequences. The presence of these MOTT highlights the care that needs to be taken when examining ancient remains for MTBC aDNA. The web-accessible 'List of Prokaryotic names with Standing in Nomenclature' listed 170 mycobacterial species in March 2015 (Euzéby, 1997). Only 62 species were known in 1989, another 27 species were identified in the 1990s and 81 have been described since 2000. This rise in newly-described mycobacteria during the last two decades indicates that the actual number of MOTT species in the environment is likely to be substantially higher than the ones known today.

Three publications have previously reported targeting of the hsp65 gene as part of studies of MTBC aDNA. Konomi et al. (2002) reported amplification of a 441 bp product from seven samples, most of these products being assigned to *Mycobacterium flavescens* based on restriction fragment length analysis. Haas et al. (2000) used a nested PCR approach giving 231 bp and 133 bp products and obtained positive results for 13 samples. Direct sequencing of the hsp65 sequences of two of the samples identified these as *Mycobacterium agri*, a saprophytic soil mycobacterium. Neither of these studies reported the actual sequences obtained from their samples but both attributed their results to contamination with soil bacteria. Finally, Crubézy et al. (1998) amplified a 383 bp segment of hsp65 from a rib and a vertebral fragment of a skeleton from Egypt dating to 3300–3400 BC. They then applied nested PCR to aliquots of the first-round PCR product of the vertebral sample and obtained amplicons of 105–135 bp which after direct sequencing gave four non-identical sequences. Further analysis of one of these nested hsp65 sequences showed that it was most similar to *Mycobacterium malmoense*, an environmental mycobacterium, and was positioned near the root of an ML tree, surrounded by a variety of MOTT species and some distance from the MTBC species (Crubézy et al., 2006). The authors then concluded that the sequence represents "an archaic mycobacteria, which was pathogenic at the origins of urban life in Egypt". We believe that this proposal is not supported by their findings and that it is more likely that the sequence derived from a contaminant rather than a true ancient pathogenic mycobacterium.

The PCRs directed at the gyrA, katG, qcrB and rpoB loci further illustrate the problem caused by the presence of non-MTBC bacteria in archaeological bones, and emphasise that false-positive PCR products can result not just from MOTT but also from bacteria of related genera that share sequence similarity within the region containing a SNP. The presence of identical sequences for a given target in different samples from various locations, as in the case of rpoB^{2646} in Ashton 118, Kempston 3902 and Wheatpieces 4 or qcrB in Ashchurch 705, Saint Amé 20, St Peter's Church 1390, and St Peter's Collegiate Church 28 and 62, might indicate similarities in the environmental microflora at these different burial sites. Another possibility is that these sequences derive from common pathogenic and/or non-pathogenic bacteria from the microbiomes of these individuals.

5. Conclusion

Our results illustrate the complications inherent in the study of MTBC aDNA in archaeological remains. The presence in such samples of a diverse microflora, a substantial proportion comprising MOTT and other species with sequence similarity to MTBC, presents challenges that have not, in our view, been met by all previous published work in the area. It is clear that the authenticity of any PCR directed at MTBC aDNA cannot be assumed unless the product is characterised by sequencing, and any variations from the reference MTBC sequence checked against sequence data for MOTT and other species to ensure that the product does actually derive from MTBC. Even if an amplicon is identical to the MTBC, authenticity

cannot be assumed, in view of the ever-increasing number of new MOTT species that are being described, which implies that many species remain unrecorded. Short sequences from conserved regions such as coding loci could quite conceivably be identical in MTBC species and one or more MOTT that might be present in an archaeological sample, as seems to be the case for the amplicon used to detect IS6110 in clinical samples (Thacker et al., 2011). Such a degree of sequence conservation between MTBC and MOTT would also complicate the use of NGS to obtain ancient pathogen genotypes or genome sequences from archaeological human samples (Bouwman et al., 2012; Chan et al., 2013; Bos et al., 2014). Misidentification of a MOTT sequence as an MTBC read would now lead to the final genome sequence being a chimera, partly authentic MTBC and partly derived from one or more MOTT. The chimeric nature of such a genome sequence would not be an issue if the misidentified MOTT reads are identical to the genuine MTBC sequences in those parts of the genome to which they map. Under these circumstances, the genome sequence will be correct, albeit for the wrong reason. A problem would arise, however, if MTBC and MOTT reads spanning a SNP were identical except for that SNP position. Now there would be a danger of the MOTT version of the SNP being called as correct, for example if the MOTT sequences dominated the read collection and the most frequent version of a SNP was taken to be the genuine read at that position. The resulting genome sequence would be chimeric in a critical way as its SNP genotype would be part MTBC and part MOTT, and deductions that are made about the evolutionary relationships of that ancient 'genome' and extant MTBC strains would clearly be erroneous.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jas.2016.03.002.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Aranaz, A., Cousins, D., Mateos, A., Domınguez, L., 2003. Elevation of *Mycobacterium tuberculosis* subsp. *caprae* to species rank as *Mycobacterium caprae* comb. nov., sp. nov. Int. J. Syst. Evol. Microbiol. 53, 1785–1789.

Baron, H., Hummel, S., Herrmann, B., 1996. *Mycobacterium tuberculosis* complex DNA in ancient human bones. J. Archaeol. Sci. 23, 667–671.

Bos, K.I., Harkins, K.M., Herbig, A., Coscolla, M., Weber, N., Comas, I., Forrest, S.A., Bryant, J.M., Harris, S.R., Schuenemann, V.J., Campbell, T.J., Majander, K., Wilbur, A.K., Guichon, R.A., Steadman, D.L.W., Cook, D.C., Niemann, S., Behr, M.A., Zumarraga, M., Bastida, R., Huson, D., Nieselt, K., Young, D., Parkhill, J., Buikstra, J.E., Gagneux, S., Stone, A.C., Krause, J., 2014. Pre-Columbian mycobacterial genomes reveal seals as a source of New World human tuberculosis. Nature 514, 494–497.

Bouwman, A.S., Kennedy, S.L., Müller, R., Stephens, R.H., Holst, M., Caffell, A.C., Roberts, C.A., Brown, T.A., 2012. Genotype of a historic strain of *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. U. S. A. 109, 18511–18516.

Brotherton, P., Endicott, P., Sanchez, J.J., Beaumont, M., Barnett, R., Austin, J., Cooper, A., 2007. Novel high-resolution characterization of ancient DNA reveals C > U-type base modification events as the sole cause of post mortem miscoding lesions. Nucl. Acids Res. 35, 5717–5728.

Chan, J.Z., Sergeant, M.J., Lee, O.Y., Minnikin, D.E., Besra, G.S., Pap, I., Spigelman, M., Donoghue, H.D., Pallen, M.J., 2013. Metagenomic analysis of tuberculosis in a mummy. N. Engl. J. Med. 369, 289–290.

Crubézy, É., Ludes, B., Poveda, J.-D., Clayton, J., Crouau-Roy, B., Montagnon, D., 1998. Identification of *Mycobacterium* DNA in an Egyptian Pott's disease of 5400 years old. C. R. Acad. Sci. III 321, 941–951.

Crubézy, É., Legal, L., Fabas, G., Dabernat, H., Ludes, B., 2006. Pathogeny of archaic mycobacteria at the emergence of urban life in Egypt (3400 BC). Infect. Genet. Evol. 6, 13–21.

Donoghue, H.D., Spigelman, M., Zias, J., Gernaey-Child, A.M., Minnikin, D.E., 1998. *Mycobacterium tuberculosis* complex DNA in calcified pleura from remains 1400 years old. Lett. Appl. Microbiol. 27, 265–269.

Donoghue, H.D., Marcsik, A., Matheson, C., Vernon, K., Nuorala, E., Molto, J.E., Greenblatt, C.L., Spigelman, M., 2005. Co-infection of *Mycobacterium tuberculosis* and *Mycobacterium leprae* in human archaeological samples: a possible explanation for the historical decline of leprosy. Proc. Biol. Sci. 272, 389–394.

Euzéby, J.P., 1997. List of bacterial names with standing in nomenclature: a folder available on the internet. Int. J. Syst. Bacteriol. 47, 590–592. List of prokaryotic names with standing in nomenclature. Available. http://www.bacterio.net (accessed 30.04.15.).

Faerman, M., Jankauskas, R., Gorski, A., Bercovier, H., Greenblatt, C.L., 1997. Prevalence of human tuberculosis in a medieval population of Lithuania studied by ancient DNA analysis. Anc. Biomol. 1, 205–214.

Gilbert, M.T.P., Hansen, A.J., Willerslev, E., Rudbeck, L., Barnes, I., Lynnerup, N., Cooper, A., 2003. Characterization of genetic miscoding lesions caused by postmortem damage. Am. J. Hum. Genet. 72, 48–61.

Gilbert, M.T.P., Binladen, J., Miller, W., Wiuf, C., Willerslev, E., Poinar, H., Carlson, J.E., Leebens-Mack, J.H., Schuster, S.C., 2007. Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis. Nucl. Acids Res. 35, 1–10.

Gutiérrez, M., Samper, S., Jiménez, M.S., van Embden, J.D.A., Marin, J.F., Martín, C., 1997. Identification by spoligotyping of a caprine genotype in *Mycobacterium bovis* strains causing human tuberculosis. J. Clin. Microbiol. 35, 3328–3330.

Haas, C.J., Zink, A., Molnar, E., Szeimies, U., Reischl, U., Marcsik, A., Ardagna, Y., Dutour, O., Pálfi, G., Nerlich, A.G., 2000. Molecular evidence of different stages of tuberculosis in ancient bone samples from Hungrary. Am. J. Phys. Anthropol. 113, 293–304.

Hershkovitz, I., Donoghue, H.D., Minnikin, D.E., Besra, G.S., Lee, O.,Y.-C., Gernaey, A.M., Galili, E., Eshed, V., Greenblatt, C.L., Lemma, E., Bar-Gal, G.K., Spigelman, M., 2008. Detection and molecular characterization of 9000-year-old *Mycobacterium tuberculosis* from a neolithic settlement in the Eastern Mediterranean. PLoS One 3 (10), e3426.

Jaffe, H.L., 1972. Metabolic, Degenerative and Inflammatory Diseases of Bones and Joints. Lea and Febiger, Philadelphia.

Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism. Academic Press, New York, pp. 21–132.

Khan, I.U.H., Yadav, J.S., 2004. Development of a single-tube, cell lysis-based, genus-specific PCR method for rapid identification of mycobacteria: optimization of cell lysis, PCR primers and conditions, and restriction pattern analysis. J. Clin. Microbiol. 42, 453–457.

Kiers, A., Klarenbeek, A., Mendelts, B., Van Soolingen, D., Koëter, G., 2008. Transmission of *Mycobacterium pinnipedii* to humans in a zoo with marine mammals. Int. J. Tuberc. Lung Dis. 12, 1469–1473.

Konomi, N., Lebwohl, E., Mowbray, K., Tattersall, I., Zhang, D., 2002. Detection of mycobacterial DNA in Andean mummies. J. Clin. Microbiol. 40, 4738–4740.

Kweon, O., Kim, S.J., Blom, J., Kim, S.K., Kim, B.S., Baek, D.H., Park, S.I., Sutherland, J.B., Cerniglia, C.E., 2015. Comparative functional pan-genome analyses to build connections between genomic dynamics and phenotypic evolution in polycyclic aromatic hydrocarbon metabolism in the genus Mycobacterium. BMC Evol. Biol. 15, 21.
Lerat, E., Ochman, H., 2005. Recognizing the pseudogenes in bacterial genomes. Nucl. Acids Res. 33, 3125–3132.
Macovei, L., McCafferty, J., Chen, T., Teles, F., Hasturk, H., Paster, B.J., Campos-Neto, A., 2015. The hidden 'mycobacteriome' of the healthy human oral cavity and upper respiratory tract. J. Oral Microbiol. 7, 10, 3402/jom.v7.26094.
Mays, S., Taylor, G.M., 2003. A first prehistoric case of tuberculosis from Great Britain. Int. J. Osteoarchaeol. 13, 189–196.
Mays, S., Taylor, G.M., Legge, A.J., Young, D.B., Turner-Walker, G.T., 2001. Paleopathological and biomolecular study of tuberculosis in a medieval collection from England. Am. J. Phys. Anthropol. 114, 298–311.
Mays, S., Fysh, E., Taylor, G.M., 2002. Investigation of the link between visceral surface rib lesions and tuberculosis in a Medieval skeletal series from England using ancient DNA. Am. J. Phys. Anthropol. 119, 27–36.
Müller, R., Roberts, C.A., Brown, T.A., 2014a. Biomolecular identification of ancient Mycobacterium tuberculosis complex DNA in human remains from Britain and Continental Europe. Am. J. Phys. Anthropol. 153, 178–189.
Müller, R., Roberts, C.A., Brown, T.A., 2014b. Genotyping of ancient *Mycobacterium tuberculosis* strains reveals historic genetic diversity. Proc. Biol. Sci. 281, 20133236.
Müller, R., Roberts, C.A., Brown, T.A., 2015. Complications in the Study of Ancient Tuberculosis. non-specificity of IS6110 PCRs. STAR 1(1), STAR2014112054892314Y.0000000002.
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.
Resnick, D., 2002. Osteomyelitis, septic arthritis, and soft tissue infection: organisms. In: Resnick, D. (Ed.), Diagnosis of Bone and Joint Disorders, fourth ed., vol. 3. W.B. Saunders, Philadelphia, pp. 2510–2624.
Roberts, C.A., Buikstra, J.E., 2003. The Bioarchaeology of Tuberculosis. A Global View on a Reemerging Disease. University Press of Florida, Gainesville, Florida.
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.
Spigelman, M., Lemma, E., 1993. The use of the polymerase chain reaction (PCR) to detect *Mycobacterium tuberculosis* in ancient skeletons. Int. J. Osteoarchaeol. 3, 137–143.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Taylor, G.M., Crossey, M., Saldanha, J., Waldron, T., 1996. DNA from *Mycobacterium tuberculosis* identified in mediaeval human skeletal remains using polymerase chain reaction. J. Archaeol. Sci. 23, 789–798.
Taylor, G.M., Goyal, M., Legge, A.J., Shaw, R.J., Young, D., 1999. Genotypic analysis of *Mycobacterium tuberculosis* from medieval human remains. Microbiology 145, 899–904.
Thacker, T.C., Harris, B., Palmer, M.V., Waters, W.R., 2011. Improved specificity for detection of Mycobacterium bovis in fresh tissue using IS6110 real-time PCR. BMC Vet. Res. 7, 50.
Tsangaras, K., Greenwood, A.D., 2012. Museums and disease: using tissue archive and museum samples to study pathogens. Ann. Anat. 194, 58–73.
Van Soolingen, D., Hoogenboezem, T., de Haas, P.E.W., Hermans, P.W.M., Koedam, M.A., Teppema, K.S., Brennan, P.J., Besra, G.S., Portaels, F., Top, J., Schouls, L.M., van Embden, J.D.A., 1997. A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: characterization of an exceptional isolate from Africa. Int. J. Syst. Bacteriol. 47, 1236–1245.
Van Soolingen, D., van der Zanden, A.G.M., de Haas, P.E.W., Noordhoek, G.T., Kiers, A., Foudraine, N.A., Portaels, F., Kolk, A.H.J., Kremer, K., van Embden, J.D.A., 1998. Diagnosis of *Mycobacterium microti* infections among humans by using novel genetic markers. J. Clin. Microbiol. 36, 1840–1845.
Wang, J., McIntosh, F., Radomski, N., Dewar, K., Simeone, R., Enninga, J., Brosch, R., Rocha, E.P., Veyrier, F.J., Behr, M.A., 2015. Insights on the emergence of *Mycobacterium tuberculosis* from the analysis of *Mycobacterium kansasii*. Genome Biol. Evol. 7, 856–870.
Wilbur, A.K., Bouwman, A.S., Stone, A.C., Roberts, C.A., Pfister, L.-A., Buikstra, J.E., Brown, T.A., 2009. Deficiencies and challenges in the study of ancient tuberculosis DNA. J. Archaeol. Sci. 36, 1990–1997.
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.
Zink, A.R., Sola, C., Reischl, U., Grabner, W., Rastogi, N., Wolf, H., Nerlich, A.G., 2003. Characterization of *Mycobacterium tuberculosis* complex DNAs from Egyptian mummies by spoligotyping. J. Clin. Microbiol. 41, 359–367.
Zink, A.R., Grabner, W., Nerlich, A.G., 2005. Molecular identification of human tuberculosis in recent and historic bone tissue samples: the role of molecular techniques for the study of historic tuberculosis. Am. J. Phys. Anthropol. 126, 32–47.