Biomolecular archaeology of ancient tuberculosis: Response to "Deficiencies and challenges in the study of ancient tuberculosis DNA" by Wilbur *et al.* (2009).

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Keywords: ancient DNA; lipid biomarkers, molecular typing; *Mycobacterium tuberculosis*; palaeopathology; PCR;

Abstract

It is sixteen years since the first detection of *Mycobacterium tuberculosis* DNA in archaeological specimens, yet the validity of findings continues to be questioned. Rigorous scientific scrutiny and debate is valuable and has led to a coalescence of procedures and precautions amongst those actively engaged in this work. It is disappointing that these good practices are not recognised by certain scientists whose primary expertise is in the related fields of archaeology, palaeopathology, and eukaryote ancient DNA. There is a danger that by constant repetition, disputable and inadequately justified concerns will assume the status of self-perpetuating myths and misunderstandings. We discuss these issues with reference to a recent article in this journal, in which clear peer-reviewed scientific data were specifically targeted as part of a general critique of the field of the palaeomicrobiology of tuberculosis. We believe we have given sufficient evidence and cogent argument to persuade the unbiased reader that the views in the critique by Wilbur *et al.* are unjustified.

1. Introduction

Writing in this journal, Wilbur *et al.* (2009) develop a systematic critique of the use of ancient DNA (aDNA) in the study of tuberculosis (TB) in archaeological specimens, focussing on an article by Hershkovitz *et al*. (2008). This paper describes TB in the Pre-Pottery Neolithic site of Atlit Yam in the eastern Mediterranean from 9000 years BP, based on osseous and biomolecular findings. The critique contains no original data and repeats many earlier criticisms of the field (Roberts & Ingham, 2008), although a detailed response to that paper has been published recently (Taylor *et al.*, 2009). The rationale of the Wilbur *et al*. (2009) critique centres

on three key arguments: (1) – rare archaeological specimens, as exemplified by Hershkovitz *et al*. (2008), are being destroyed for scientifically unjustified reasons; (2) – there are validated reliable osseous changes, superior to those described in the Atlit Yam study, which should be utilised prior to any examination for aDNA; and (3) – the DNA extracted is most likely from sources rather than the aDNA from *Mycobacterium tuberculosis.* Wilbur *et al*. (2009) argue the necessity of using specified facilities to avoid modern contamination; the need to clone sequences rather than relying on direct sequencing; question the specificity of polymerase chain reactions (PCRs), the likelihood of amplifying and misinterpreting sequences derived from environmental microorganisms and the difficulties of interpreting negative results. Summarising their concerns, they state – "Archaeological remains are non-renewable resources and, in the context of TB research, should not be destroyed on the basis of a questionable palaeopathological assessment". They then comment that their views are broadly applicable within biomolecular palaeopathology and more generally in aDNA research as a whole.

2. Destructive analysis – is it necessary and to what extent?

Wilbur *et al.* (2009) assert that whilst a sample of less than 500 mg is sometimes sufficient to indicate the presence of TB in a specimen, in order to be able to determine the strain of *M. tuberculosis* it is necessary to take two 2 g samples, to enable duplicate analysis. These samples should be from a region of bone adjacent to a pathological lesion and, along with a similar-sized sample from a non-tuberculous skeleton acting as a negative control, from the same archaeological site. They cite no peer-reviewed articles to support this assertion but state it is based on their experience, which appears to be limited (Table 1). Samples of this size are indeed

destructive and the authors query the usefulness of destructive analysis for verification of TB in skeletons of increasing antiquity as this is "scientifically meaningless" and cannot be justified.

2.1 Necessity for destructive sampling?

A review of those papers on *M. tuberculosis* aDNA, which indicate the amount of sample removed (Table 1), shows that it is not necessary to take such large samples. Particular investigators appear to have a preference for the size of sample used, but this seems not to influence the outcome. Most studies have not sought to distinguish between the individual members of the *M. tuberculosis* complex. This group of genetically similar organisms includes the principal human pathogen *M. tuberculosis*, the less common *Mycobacterium africanum*, *Mycobacterium canettii* and the species that primarily infect animals: *Mycobacterium bovis*, *Mycobacterium microti* and *Mycobacterium caprae*. The majority of studies used between 25–400 mg of sample, which was sufficient for molecular characterisation and determination of genetic lineages of the *M. tuberculosis* aDNA. In our experience, better results are obtained with a small quantity of sample, due to the problem of PCR inhibition. The quantity of *M. tuberculosis* aDNA in specimens will vary but positive findings have been reported from 'a few mg', 'surface scrapings', material from 'the inner part of bones' and the dental pulp region of teeth. Researchers take small and careful samples precisely to avoid the level of destructive sampling that Wilbur *et al*. (2009) are seeking to impose on the field.

2.2 Use of samples from skeletons without lesions as negative controls

The suggestion that bones without lesions from the same archaeological sites as those with bony changes suggestive of TB, should be used as negative controls is scientifically flawed. It is generally agreed that only approximately 3–5% of people with TB will develop bone changes. Therefore, the overwhelming majority of infected people will have none. In the pre-antibiotic era tuberculosis was an endemic, if not an epidemic disease. Therefore, supposedly negative skeletons from archaeological sites, that include some individuals with bony changes, are also very likely to have been infected in life. Indeed, it has been proved that many such skeletons do contain biomolecular markers of TB (Baron *et al*., 1996; Faerman *et al.*, 1997; Mays *et al.* 2002; Fletcher *et al*., 2003a; Zink *et al*., 2001). When TB was first reported in skeletons without pathology, there was justifiable scepticism and positive findings were suspected to be due to laboratory contamination; we now appreciate that these data are entirely consistent with the pathology of TB (Donoghue & Spigelman, 2006).

In our laboratories we use negative extraction controls, which are sham-inoculated and processed in parallel with the actual specimen samples. This indicates whether reagents are contaminated, or whether cross-contamination has occurred. Taking 2 x 2 g samples from skeletons with no bony lesions from the same site as skeletons with lesions will not be helpful for negative controls. However, it could be useful in providing additional data on the extent of TB infection at that site.

2.3 Interpretation of negative findings

One of the reasons that the critique co-authors cite for the lack of justification for further molecular analysis of material, of increasing antiquity, is that negative findings cannot be taken

as evidence of absence of TB. We have emphasised previously (Spigelman & Donoghue, 2003) that absence of proof is not proof of absence, and Taylor *et al*. (2009) discuss the topic in their response to Roberts & Ingham (2008). False negatives may be due to poor preservation of the TB biomarkers. However, in our experience PCR inhibition is also a very common phenomenon. This can be readily demonstrated using an inhibition assay and real-time PCR, as it is indicated by an increase in cycle threshold when compared with the uninhibited control (Nolan *et al.*, 2006). This is why the Atlit Yam study included an additional biomolecular marker of TB – *M. tuberculosis* complex-specific mycolic acids (*vide infra*). This overcomes both of the principal causes of false negative findings based on aDNA.

2.4 Studies of prehistoric material are not "meaningless"

Wilbur *et al.* (2009) assert that "there appears, in our opinion, to be no justification for further destructive analysis of material for molecular verification of TB in skeletons of increasing antiquity that may or may not exhibit lesions 'typical' of the disease (e.g. Hershkovitz *et al*., 2008). Such use of destructive analysis to search for 'the world's oldest cases of human tuberculosis' … is scientifically meaningless…". However, we believe that most of the readers of this journal would be interested in whether early farming communities in the Levant had TB, especially in a site that supplies clear-cut evidence of cattle domestication. This is an important fundamental question in studying the dynamic of this Agro-Pastora-Marine population, people who paved the road to modern society and the Mediterranean mode of subsistence. The Atlit Yam study was not a hunt for the "oldest case" of tuberculosis but aimed to shed light on key questions regarding the health of early coastal communities in the Levant.

2.5 M. tuberculosis aDNA and phylogenetic studies

Another reason given by Wilbur *et al*. (2009) for the lack of justification for further analysis of material of increasing antiquity for molecular verification of TB is because the exercise is, "scientifically meaningless as we know that the bacterium has been in existence for longer than *H. sapiens*". This refers to phylogenetic studies of contemporary *M. tuberculosis* complex strains which indicate that the *M. tuberculosis* progenitor strain was present approximately 2.5–3.0 million years ago (Gutierrez *et al.,* 2005). The critique authors continue, "its simple detection in human skeletons of increasing antiquity tells us nothing of value about the existence of the disease in the past".

The comments on the use of ancestral sequence inference and estimates of Most Recent Common Ancestor (MRCA) ignore the fact that such estimates require validation. There are wide variations in such estimated MRCAs and usually more than one possible interpretation of the data. It is well known that phylogenetic inferences can be biased and may be inaccurate even with strong bootstrap or posterior support (Lunter, 2007). Working models are necessary and are revised as additional data become available (Gordon *et al.,* 2009). Indeed, molecular archaeology has been identified as a possible source of information on *M. tuberculosis* strains that pre-date the current MRCA (Smith *et al*., 2009).

The detection and identification of a strain of *M. tuberculosis* that lacked the TbD1 deletion region (Hershkovitz *et al.,* 2008) is of interest because Atlit Yam was one of the earliest sites known where there was animal domestication but no dairying. The emergence of the major lineage of TbD1-deleted strains is a significant event whose date has been estimated by

phylogenetics (Wirth *et al.,* 2008). The detection of an *M. tuberculosis* TbD1-deleted strain from 9000 years BP enables a marker in real time to be included in the estimation of MRCA. In addition, the finding of the human pathogen *M. tuberculosis* in a community with large numbers of cattle, goats, sheep and pigs provides direct evidence that the first farmers were not infected with *M. bovis,* which had been inferred previously from a comparison of the genomes of these two species (Brosch *et al.,* 2002).

3. Interpretation of skeletal evidence

A major concern of Wilbur *et al*. (2009) "is the sometimes naïve and incomplete use of skeletal information to provide evidence that bones come from an individual who suffered from and/or was exposed to the disease." Again, the destructive analysis of rare and irreplaceable material is mentioned as the unjustified outcome of such naïve analyses. They give a useful overview of the pathognomonic and non-pathognomonic indicators of TB, but object to the endocranial labyrinth-type lesions, termed *serpens endocrania symmetrica* (SES), found in the Atlit Yam study being described as associated with TB.

3.1 The 'rare' argument and destructive analysis

Since Wilbur *et al.* (2009) do not define "rare specimen", their key parameter, nor how this is assessed, we cannot argue with this claim – instead we present the facts. More than 65 skeletons have been found and reported from Atlit Yam (Galili *et al*., 1993, 2005, 2008; Hershkovitz & Edelson, 1991; Hershkovitz & Galili, 1990, 1991; Hershkovitz *et al*., 1991a,

1991b). Besides the mother and the child skeletons, representing most of the bones, at least two other individuals from this site show osseous changes that suggest TB. More important, no serious damage was caused to the bones of the mother and the child, as only very small fragments were removed (Table 1). There is sufficient osseous material from the mother and the child and other skeletons from the site for many future studies. In addition, there are hundreds of Pre-pottery Neolithic skeletons from the southern Levant that are available for investigation. Every archaeological investigation requires sacrificing some material or data in order to gain crucial information and knowledge. Responsible archaeologists use the best available technology at the time of the research and leave sections of sites and some of the archaeological material for future generations. That is exactly what was done in Atlit Yam, where the antiquities and the data are at immediate risk of destruction because of natural exposure by marine erosion. Therefore, the criticisms by Wilbur *et al.* (2009) are unjustified and it is misleading for them to imply that they are more concerned for the preservation of the Atlit Yam skeletons than are the scientists, who have excavated and studied them for more than 15 years.

3.2 Reliable bone criteria for TB

Here the critique authors appear to contradict themselves. On one hand they claim that aDNA analysis should be avoided unless there is solid evidence based on bone palaeopathology for the presence of TB (thus excluding 97% of cases). On the other hand they rightly state that, "The critical issue when considering skeletal evidence for TB is to be aware that there are no pathognomonic lesions – those that can be considered specific to TB – and rather we are left with lesions that are 'consistent with' a diagnosis of TB but also consistent with other conditions."

They then state that as a consequence, a differential diagnosis is always required. We prefer to conclude that TB can be confidently diagnosed in ancient bones only on the basis of biomolecular investigations such as a study of aDNA or other biomarkers. Wilbur and colleagues (2009) next criticise the osseous parameters, used in our study to suggest the possibility of TB in the Atlit Yam population.

3.2.1 Serpens Endocrania Symmetrica (SES)

The SES endocranial labyrinth-type lesions are beyond X-ray resolution so cannot be detected on radiographs or even CT images, which is why this criterion does not appear in medical records. However, SES was recognised in 32 of 1,884 adult skulls (1.7%) from the Harmann-Todd (HTH) collection housed at the Cleveland Museum of Natural History (Hershkovitz *et al*., 2002). The frequency of SES among individuals with tuberculosis as the cause of death in their medical records was 4.4%, and this included 25 of the 32 individuals (78.1%) with SES. The rate of SES in the non-TB sample was only 0.53%. These numbers are better by far than any other osseous indicators in suggesting the presence of intra-thoracic inflammation at the time of death. Furthermore, there are earlier macroscopic and microscopic studies that indicate a clear relationship between SES and TB (e.g. Schultz, 1999, 2001).

Considering SES in the skull of children, Wilbur *et al*. (2009) confirm that: “we have observed that a large number of infant crania demonstrate such lesions, and the published literature supports these observations”. We note that since they do not supply their own data, Hershkovitz *et al*. (2002) is cited as supportive evidence. In this study of 40 children aged 1 to 15, from the HTH collection, three had typical SES lesions, of whom two had medical records that they died of TB. The study by Lewis (2004), cited to illustrate that endocranial lesions are a common phenomenon among children, was based on sub adult skeletal samples from historical

British cemeteries, and no medical records to verify the cause of death were available, making the distinction between normal and pathological growth difficult. Interpretation of the data, therefore, was necessarily subjective.

We wish to emphasise that SES is probably due to circulatory hypertension and is an osseous change consistent with tuberculosis but not pathognomonic. We have never stated that SES is associated with meningitis tuberculosa. Thus it is misleading of Wilbur *et al*. (2009) to write a whole paragraph on "the course of meningitis tuberculosa…" in association with SES, in a discussion of our paper under the heading "An example of over-interpretation". It is precisely because SES is not diagnostic for TB that additional studies were undertaken. We note with interest that, in a recent publication (Roberts *et al.,* 2009), one of the Wilbur *et al.* (2009) co-authors (CAR) views SES as worthy of further study (e.g. by biomolecular methods for TB) in order to assess the range of conditions that may cause the lesions described.

3.2.2 Hypertrophic Osteoarthropathy (HOA)

It is well recognised that intra-thoracic and pulmonary diseases are linked with HOA (Mays & Taylor, 2002; Resnick & Niwayama, 1995; Rothschild & Rothschild, 1998) and SES is associated with HOA (68.0% of SES individuals also had HOA). The critique authors urge palaeopathologists to consider differential diagnoses for the lesions observed, determine their most likely cause and publish detailed descriptions in supplementary data to journal articles. However, in the Hershkovitz *et al.* (2002) study, differential diagnoses for SES and HOA were presented and discussed. What is the logic of repeating it each time we use these parameters? We prefer to use other biological markers that can answer the question directly.

3.2. 3 Strategy to be used in palaeopathological studies

The apparent conclusion in Wilbur *et al*. (2009) is that it is unjustified to carry out biomolecular investigations on bones that have changes consistent with TB but which are not diagnostic. They describe the presence of destructive lesions in the lower thoracic and/or lumbar vertebrae, especially on the anterior aspect of the vertebral body, as diagnostic for TB, yet appear to be unaware that one of the individuals from Atlit-Yam shows collapse of the anterior aspect of a thoracic vertebra (Hershkovitz and Galili, 1990). Other than this, they do not tell us what they regard as legitimate criteria for identifying TB in ancient bones other than citing two medical books (Jaffe, 1972; Resnick & Niwayama, 1995).

Having concluded that Hershkovitz *et al*. (2008) used no legitimate criteria for the identification of TB, this study is cited as an example where biomolecular investigation was not justified in the light of the palaeopathology. However, as discussed above in Section 2.2, bones with no signs of morphological changes have been found positive for *M. tuberculosis* complex aDNA and Zink *et al*. (2001; 2003b) found 14-15% of their control group to be positive. We argue that the Atlit Yam Neolithic study was justified by the outcome; the bones showed changes consistent with a diagnosis of TB and *M. tuberculosis* aDNA was detected, with additional molecular characterisation. Finally, TB infection was confirmed, independently and without amplification, by the detection of TB-complex specific mycolic acid lipid biomarkers. A clear conclusion from Hershkovitz *et al.* (2008) is that skeletal evidence alone may be insufficient to diagnose TB and that confirmation may be obtained by co-ordinated analyses of molecular biomarkers.

4. Specificity of biomolecular evidence

The critical points under this heading are that unlike clinical samples, archaeological bone may have many environmental mycobacteria present, which will cause problems in differentiation from the *M. tuberculosis* complex. Therefore, unmodified clinical tests should not be used until their specificity has been determined. Questions are raised about PCRs, which target the insertion elements IS*6110* and IS*1081*, based on their distribution and lack of sequence variation. The use of single nucleotide polymorphisms (SNPs) is queried, because they may occur in genes found in environmental mycobacteria and therefore co-amplify and give rise to misleading results. Finally, the usefulness of spacer oligotyping (spoligotyping) based on the Direct Repeat (DR) region of the organisms that comprise the *M. tuberculosis* complex (Kamerbeek *et al.,* 1997) is questioned, because of poor reproducibility and variation caused by recombination and insertion events mediated by the IS*6110* element.

4.1 Specificity of PCRs for M. tuberculosis *aDNA*

There are plentiful data on the specificity of PCRs designed for clinical use to detect members of the *M. tuberculosis* complex, and individual species within this group. It is indeed correct that the earliest studies on archaeological *M. tuberculosis* (Spigelman & Lemma, 1993), including one with a critique co-author (JB) (Salo *et al.,* 1994), used the methodology devised for clinical diagnosis based on IS*6110*. The primers that were selected (Eisenach *et al*., 1990) are specific for the *M. tuberculosis* complex and do not amplify "mycobacteria other than tuberculosis" (MOTTs). MOTTs include well-recognised opportunistic pathogens that are always considered during the development of diagnostic tests. Due to their increasing importance

in patients with a reduced immune response, there are PCRs designed to target these organisms and to distinguish them from the *M. tuberculosis* complex.

In all PCRs used for aDNA, the length of target region is a prime concern due to the likelihood of DNA fragmentation. Therefore, primers have been modified where necessary for aDNA work. In addition, the reactions themselves are optimised to allow for the inhibitors that are often present (Taylor *et al.* 2009). It is incorrect to imply that tests are taken "off the shelf in a clinical setting". The IS*6110* element can occur up to 25 copies/cell of *M. tuberculosis*. This increases the likelihood of detection and this locus is the most widely used. As it is entirely absent in a minority of modern strains and is only present as a single copy in *M. bovis*, PCRs based on IS*1081* have also been applied to aDNA, as this is present at 6 copies/cell in all members of the *M. tuberculosis* complex, including *M. bovis* (Taylor *et al*., 2007).

Wilbur *et al.* (2009) repeat the unsupported assertion that it is essential that PCR products are cloned and sequenced to ensure that a positive result is due to the actual target locus and not one of its homologues in an environmental mycobacterial strain. This ignores the fact that *M. tuberculosis* PCR methods are highly specific. It also ignores the additional hazards introduced by cloning, such as replication errors caused by the *Taq* polymerase (Eckert & Kunkel, 1991) – a special problem for repetitive sequences, and the physical containment problems posed by the production of large quantities of the amplified material (Taylor *et al.*, 2009). There is experimental evidence from a study of *Mycobacterium leprae* (Taylor *et al.*, 2006), produced by one of the Wilbur *et al.* (2009) co-authors (AB), that cloning gave no added value to data obtained by direct sequencing, but did introduce some errors, which were ascribed to *Taq* polymerase error and slipped strand mispairing.

4.2 Further characterisation of M. tuberculosis aDNA

The *M. tuberculosis* complex members show more than 99.95% nucleotide sequence similarity and a strictly clonal population structure (Smith *et al.*, 2009). They can readily be speciated and typed by deletion analysis. The TbD1 locus mentioned in Section 2.5 above marks a fundamental division in *M. tuberculosis* so to determine whether the aDNA is from a TbD1-intact or TbD1-deleted strain is of phylogenetic and palaeogeographical significance (Wirth *et al.*, 2008). However, Wilbur *et al.* (2009) show no sign that they appreciate its importance. In discussing the detection of the TbD1 deletion in aDNA they merely make the obscure comment that "the test is not discriminatory for *M. tuberculosis* as both outcomes can be interpreted the same way." Further, they assert that the two insertion elements and the TbD1 deletion "cannot be used if biomolecular study of ancient TB is to contribute to understanding of the evolution of the bacterium and the disease." We disagree.

The use of SNPs is next discussed. The authors raise concerns because SNPs may occur in genes found in environmental mycobacteria and therefore co-amplify and give rise to misleading results. This is indeed a possibility so the primers have to be carefully designed. It is easier to minimise extraneous co-amplification if genetic loci are chosen that are either unique to the *M. tuberculosis* complex, or have been well characterised in a large number of other species. In order to obtain SNP data, the samples have to be chosen with care, because PCRs will be based on a single copy genetic locus, and good preservation is necessary. Samples should be selected that give clearly positive results in screening tests. *M. tuberculosis*-negative samples from the same population can provide negative controls for the SNP PCRs.

4.3 Spoligotyping

This technique, which is used as an international typing system, makes use of polymorphism in the Direct Repeat (DR) region found in the *M. tuberculosis* complex, where unique spacer regions separate identical DR sequences. Using primers for the DR region, one of which is labelled, a PCR is performed and the resulting mix of amplicons hybridised with the appropriate spacer oligonucleotide on a membrane in a reverse dot blot procedure (Kamerbeek *et al*., 1997). This technique has been applied to archaeological TB studies because each DR and spacer is only 50-60 bp in size, so data can be obtained even if the DNA is fragmented (Taylor *et al*., 1999). In clinical studies the technique is used to distinguish between strains and to indicate short-term epidemiological trends. The Atlit Yam study obtained spoligotyping data, but these were of limited use beyond providing data from an additional genetic locus. This is because it appeared that all spacer regions were present. Spoligotypes were obtained from two specimens and repeated in triplicate. There was some variation between the repeated reactions, attributed to poor preservation, but no evidence of any deleted spacer. There is a unidirectional loss of spacers, and modern clinical practice appears to have accelerated this process, shown by a comparison between the 18th century spoligotypes obtained from naturally mummified human remains from Hungary (Fletcher *et al*., 2003b) and those now prevalent in Western developed countries. Therefore, spoligotypes with no loss of spacers are correctly described as "ancestral", although examples still occur today. Wilbur *et al*. (2009) assert that it is wrong to describe spacer loss as unidirectional because of recombination events facilitated by IS*6110*. They summarise the role of spoligotyping in aDNA as being "not helpful in studies of ancient TB." IS*6110* certainly plays a role in the internal re-arrangement of the genome (Warren *et al*., 2002), but

there is no evidence of the re-acquisition of deleted spacers, so the criticism is unjustified. The insertion of IS*6110* can mask the presence of spacers, but is far more likely to cause large-scale deletions by double recombination events. We note that one of the Wilbur *et al*. (2009) senior co-authors (CAR) enthuses about the usefulness of deletion analysis, SNP typing and spoligotyping on her website (Roberts, 2009), positively mentioning some of our earlier work. This indicates a complete reversal of opinion to that in the critique by Wilbur *et al*. (2009).

5. Biomolecular evidence must be rigorous

The appropriate procedures to use in aDNA research have been debated since its inception, and disagreement continues between those working on eukaryote aDNA and those who study the aDNA of microbial pathogens. Put simply, the extreme positions are between those who believe no work should be performed without dedicated high-containment facilities, and those who believe that good molecular biological practice, with a spatial and temporal separation of activities, is appropriate. Wilbur *et al*. (2009) suggest that the "TB aDNA community" makes two underlying assumptions, which if correct, would lessen the burden of authentication compared with, for example, equivalent research with human aDNA. These are that mycobacterial aDNA is more resistant to degradation than aDNA from other sources; and that laboratory contamination with modern DNA is less of a problem than when human aDNA is studied. The authors then suggest that the "TB aDNA community" might use these assumptions as an argument for taking less stringent anti-contamination control measures and for being less rigorous in asking if an amplicon derives from aDNA rather than a contaminant. We are happy to put the record straight.

5.1 Is the preservation potential of mycobacterial aDNA greater than that of aDNA from other organisms?

The critique authors argue that there is no proof that the mycobacterial hydrophobic cell wall retards DNA decay, although they comment that during life the unusual cell wall "clearly confers an additional degree of protection on the internal contents". Unfortunately, they base their stance on an internet "Textbook of Bacteriology" (Todar, 2009), which lacks essential quoted references and is factually incorrect in its description of the mycobacterial cell wall, totally ignoring the mycolyl arabinogalactan, and pathologically important free lipids, such phthiocerol dimycocerosates. Todar (2009) provides no basis for Wilbur *et al.* (2009) entering into any serious discussion of cell wall function in *M. tuberculosis*, but it appears that the authors are trying to argue that *M. tuberculosis* aDNA lacks special protection and its analysis should not be subject to special rules, different from human aDNA.

The key facts are that *M. tuberculosis* aDNA is not analysed less rigorously than human aDNA but, since it can be reliably retrieved from archaeological material (Table 1), it is accepted scientific practice to suggest some explanation. The demonstration of the persistence of *M. tuberculosis*-specific cell wall lipid biomarkers (Donoghue *et al.*, 1998; Gernaey *et al*., 1998, 2001) indicated that the integrity of the mycobacterial cell wall may play a role in the protection of the cell contents, including the DNA. This was supported by the observation that *M. tuberculosis* complex aDNA is occasionally detected in specimens where no human DNA can be amplified (Haas *et al.*, 2000; Zink *et al.*, 2005a). Zink *et al.* (2003a) commented that "mycobacterial species are better preserved than other bacteria due to their lipid-rich cell wall:

this enhances the chances of detecting enough intact mycobacterial aDNA fragments necessary for a positive analysis." Similarly, Spigelman & Donoghue (2003) proposed, citing appropriate references, that that the persistence of mycobacterial aDNA results from a combination of factors. These included the unusually low permeability of mycobacterial cell walls, but also the capacity of prokaryotes to resist DNA damage at times of stress, such as during the early period of host autolysis and the sequestration of microbial DNA in a chemically stable conformation within the cell (biocrystallisation). Wilbur *et al*. (2009) propose that "nuclease enzymes, water and free radicals, already present in the bacterium" are the prime movers for DNA degradation but this fails to explain the persistence of viable *M. tuberculosis* after the death of the host, and the ability of the organism to enter a prolonged persistent state, possibly via endospores (Ghosh *et al*., 2009). In addition to the protection given by the cell wall, we have suggested that mycobacterial DNA is intrinsically more stable than mammalian DNA because it is rich in guanine (G) and cytosine (C). We know that G:C pairs are more thermally stable than adenine (A) and thymine (T) pairs (Wada & Suyama, 1986), as G:C pairs are connected by three hydrogen bonds and A:T pairs by two. Therefore, the stronger link between the DNA strands in the GC-rich mycobacteria may also aid its persistence.

It is true that whilst our suggested mechanisms for *M. tuberculosis* aDNA persistence are based on published observations and possible mechanisms, there is no experimental evidence that proves whether they are correct. However, the important point, we must emphasise, is that we do not then extrapolate our working assumption, to the point where we take fewer or no precautions to protect the validity of our work. Based on published observations, we believe that just because human aDNA cannot be amplified in some specimens, it is unjustified to assume that detection of *M. tuberculosis* aDNA must be due to a laboratory artefact.

5.2 Is laboratory contamination less of a problem when mycobacterial aDNA is being studied?

The authors query the belief of Donoghue & Spigelman (2006) that laboratory contamination with modern DNA is less of a problem for work on *M. tuberculosis* aDNA than human DNA. Indeed, they state that the opposite is true and that modern contamination is a more difficult problem when TB is being studied. However, no modern *M. tuberculosis* DNA is ever used in our clean and PCR set-up laboratories. Referring to the Hershkovitz *et al*. (2008) paper, which is again criticised on this point, one of the centres involved in the study used a brand new laboratory, where no DNA extractions or PCRs had ever been performed previously. We agree that cross-contamination, with amplicons from previous PCR experiments, is a major concern in all PCR work. This is why we have strict separation of work in different laboratories. However, we think it unwise for the authors to disregard the ease with which reagents and extracts may become contaminated with human DNA. A recent study, carried out at University College, London, demonstrated that while it is difficult to find evidence of contamination of human gene sequences in tubes left exposed to the air in laboratories, or even a busy office, it is not uncommon for reagents to contain human DNA biomarkers, even when freshly-purchased from the manufacturer (Witt *et al*. in press).

5.3 Required standards for biomolecular studies of M. tuberculosis *aDNA*

The authors return to their well-known stance, that the use of clean rooms with filtered air

supplies and restricted access "is the only way to reduce the possibility of amplicon cross-contamination to the extent where it is possible to have confidence that results obtained with ancient specimens are genuine." If this assertion is taken to its logical conclusion, it implies that every hospital diagnostic laboratory and every research facility that lacks these features is producing questionable data. We have to emphasise that we can obtain valid data, backed up with plentiful negative DNA extraction and amplification controls, without the need for a dedicated facility, with containment and filtered air. The comments made by Taylor *et al*. (2009) on this topic are sensible and we agree with them. The precautions we take are common to all laboratories involved in the detection of DNA in samples, including those carrying out clinical diagnostic work, where the outcome can be of vital importance to the correct treatment of patients. The authorities cited by the authors are based on human aDNA work, and our earlier response (Donoghue & Spigelman, 2006) was an attempt to bring the benefit of our on-going practical experience of *M. tuberculosis* and other microbial aDNA into the debate.

Wilbur *et al*. (2009) next return to their assertion that cloning of amplified PCR sequences is essential in order to detect miscoding errors, specifically C to T transitions, which, they argue, would provide evidence of the age of the DNA. Their argument is based on a large-scale sequencing study of DNA from bison, humans and Eurasian cave lions (Brotherton *et al*., 2007). However, there is no discussion about whether this observed transition occurs in prokaryote GC-rich DNA. We refer the reader to our earlier comments on cloning in Section 4.1 above. The advantage in relying on direct sequencing is that the data obtained will be from the predominant sequence. Any minor components with sequence variations, which may be caused by replication errors, cloning errors, etc., will not be detected unless they occur in an early cycle of amplification. Wilbur *et al*. (2009) interpret the contrast in quality between the DNA sequence

electrophoretograms in Hershkovitz *et al*. (2008) as an indication that the better sequence from the TbD1 locus was derived from cross-contamination with a previous PCR product. Clearly they disregard our repeated descriptions of the precautions taken and the laboratory separation of the extraction, amplification and post-PCR activities. We postulate that the TbD1 locus may be better preserved because of its site on the genome and possible secondary or tertiary DNA structure. In addition, the PCR target locus bridges a deletion, so it is exquisitely specific for the *M. tuberculosis* TbD1-deleted lineage (Huard *et al*., 2003). Other potential reasons for the poor sequence of the conserved membrane protein amplicon are that there may be some shared homology with environmental strains, or an early replication error in the PCR reaction. However, overall, the findings of Hershkovitz *et al*. (2008) make phylogenetic sense. There is palaeopathology indicative of an infectious disease that may be TB, there are aDNA data from five genetic loci that are consistent with a finding of the *M. tuberculosis* complex and of a particular lineage of *M. tuberculosis*, together with a negative result from a genetic locus specific for *M. bovis*. However, in order to answer the question conclusively, the authors relied on a distinct third pillar of evidence – the direct detection of *M. tuberculosis*-specific cell wall lipid biomarkers.

5.4 Independent verification by an unrelated technique – lipid biomarker analysis

The problems of aDNA research resolve around its long-term stability and the need for its amplification, which creates additional concerns due to the possibility of contamination. Although these can be resolved with due care, it is undisputed that DNA may be poorly preserved in some specimens, even where there is convincing TB palaeopathology. The use of

M. tuberculosis complex-specific mycolic acid lipid biomarkers has been investigated in a limited number of studies (two of which include a critique co-author – CAR) (Donoghue *et al*., 1998; Gernaey *et al*., 1998, 2001; Hershkovitz *et al*., 2008). Another class of mycobacterial lipids, the mycocerosic acids, has also recently been shown to be suitable biomarkers for TB diagnosis in archaeological human remains (Redman *et al*., 2009). This latest study, which includes the critique co-author CAR, concludes that, "lipid biomarkers, such as mycocerosic and mycolic acids, are particularly robust and are ideal in complementing detection of ancient DNA." It is therefore very difficult to understand why there is not one reference in the Wilbur *et al*. (2009) critique to the mycolic acid data in Hershkovitz *et al*. (2008). As described in Sections 2.3 and 3.2.3 above, some of the queries raised in the critique are answered by the detection and confirmation of these lipid biomarkers.

6. Conclusions

There is no doubt that criticism and debate are normal and essential phenomena in the scientific process and crucial for the scientific progress. Such discussion should be conducted with a mind open to persuasion in the light of scientific data, not on a partial assembly of facts and unsupported assertions. We believe we have given sufficient evidence and cogent argument to persuade the unbiased reader that the pessimistic tone of the critique by Wilbur *et al*. (2009) is unjustified. We recognise that some of the critique authors have global recognition in their fields, but they lack necessary expertise in microbial aDNA. Thus the attempt to impose their views on the "TB aDNA community" appears to be based on their opinions rather than any relevant practical experience. The field of palaeo- or archaeological microbiology is exciting and

becoming more so, with the development of the technologies that will enable studies going even further back into the past, based on robust biomarkers such as the different classes of *M. tuberculosis* cell wall lipids, and proteins. The role of aDNA in validating microbial phylogenomic studies is becoming recognised and these two branches of science are reaping the benefit of closer cooperation. We trust that this frank exchange of views highlights the challenges of this field and will encourage others to participate in this exciting research arena.

Acknowledgements

The CARE, MAFCAF and Dan David Foundation supported the archaeological and anthropological work. A grant from The Leverhulme Trust (F/125/AK) for the work on lipid biomarkers is acknowledged; DEM benefited as a Leverhulme Emeritus Fellow. MS is funded by the Deutsche Forschungsgemeinschaft (DFG) for a trilateral project between German, Israeli and Palestinian researchers. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of Hershkovitz *et al*. (2008) or this manuscript.

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