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Lipid biomarkers provide evolutionary signposts for the oldest known cases of tuberculosis

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SUMMARY

Studies on the evolution of tuberculosis, and the influence of this disease on human and animal development and interaction, require the accumulation of indisputable biomarker evidence. Ideally, the determination of full genomes would provide all the necessary information, but for very old specimens DNA preservation may be compromised and only limited DNA amplification may be a possibility. *Mycobacterium tuberculosis* is characterised by the presence of unusual cell envelope lipids, with specific biomarker potential. Lipid biomarker recognition has been decisive in pinpointing the oldest known cases of human and animal tuberculosis; the former are a woman and child from a pre-pottery settlement at Atlit-Yam, Israel (~9,000 ka) and the latter is an extinct *Bison antiquus* from Natural Trap Cave, Wyoming (~17,000 ka). Including some new data, it is demonstrated how analysis of a combination of mycolic, mycocerosic and mycolipenic acid and phthiocerol biomarkers provide incontrovertible evidence for tuberculosis in these landmark specimens.

1. Introduction

Exploration of the origins and evolution of tuberculosis necessarily relies on the clear unambiguous identification of ancient well-characterised archaeological specimens. It would be advantageous to identify an extended population of infected individuals but, given the likely scarcity of the oldest examples, investigation of single landmark cases may well be a productive option. If studies of isolated cases are well-conducted and published in established peer-reviewed journals, the results achieved must be properly respected. In this report, the incontrovertible evidence for tuberculosis in the oldest human [1] and animal [2] cases will be reviewed and some new data included.

The widest possible combination of complementary methods should be used to diagnose ancient mycobacterial disease. For skeletal material, considerable expertise has been developed in recognising characteristic bone changes linked to tuberculosis infection [3,4]. The precise diagnosis of tuberculosis disease requires recognition of decisive biomarkers [5] for the causative agent *Mycobacterium tuberculosis*. During the past twenty years, DNA fragment analysis has been extensively utilised [5]. Major advances in determining full genomic data have been recently provided by the application of so-called “Next Generation Sequencing” [6] and the more direct “Metagenomic” approach [7].

The predominant feature of the tubercle bacillus is the presence of high proportions of long-chain lipids, easily distinguishable from any mammalian lipids. In a pioneering study, the 70 to 90 carbon mycolic acids (MAs) (Figure 1A) were clearly identified in a mediaeval bone from Addingham, UK, complementing DNA

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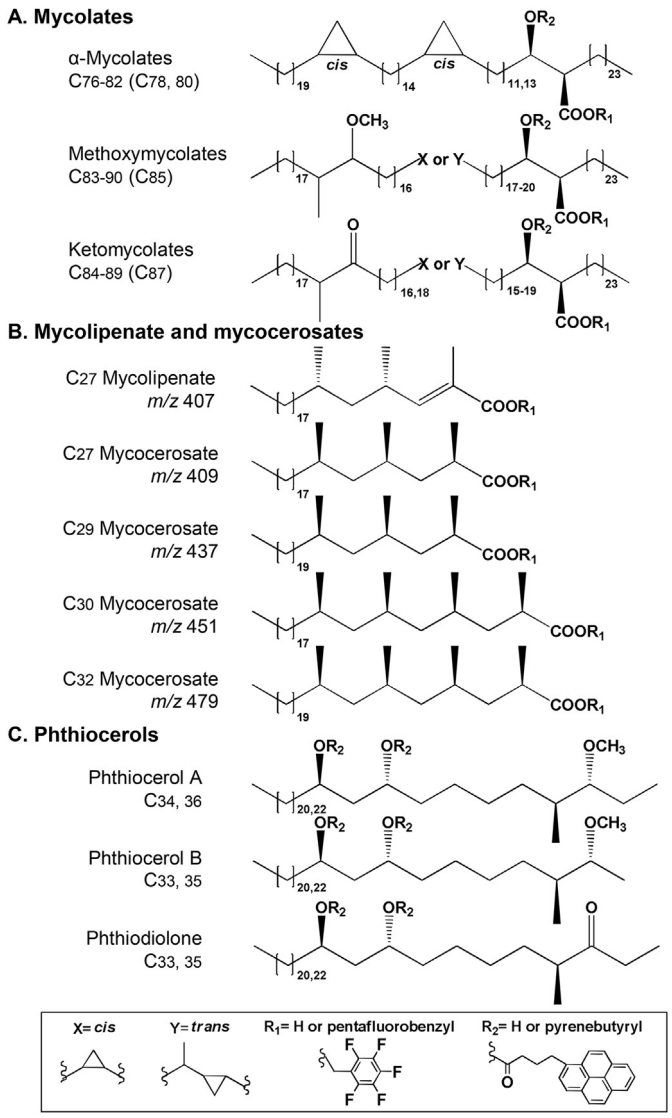

Figure 1. Lipid biomarkers for *M. tuberculosis*. (A) Generalized structures of α-, methoxy- and ketomycolates; the main components are in brackets. (B) Structures of mycolipenate and mycocerosates, showing negative carboxylate ions used for selected ion monitoring on NI–CI GC–MS analysis of pentafluorobenzyl (PFB) esters. (C) Structures of members of the phthiocerol family.

amplification and skeletal indications [8]. The biomarker range has been extended to include multi-methyl-branched mycocerosic and mycolipenic acids (Figure 1B) [2,5]. MAs were originally analysed by fluorescence High Performance Liquid Chromatography (HPLC) of slightly unstable methylanthryl esters [8], so a special robust derivatisation protocol, involving pyrenebutyrates of pentafluorobenzyl (PFB) esters was systematically developed [1,2,5]. The mycocerosate and mycolipenate PFB esters can be exquisitely detected by Selected Ion Monitoring (SIM) Negative Ion Chemical Ionisation (NI–CI) Gas Chromatography Mass Spectrometry [2,5]. Lipid biomarker detection has been particularly decisive in diagnosing tuberculosis in ribs from a woman and child from a pre-pottery settlement at Atlit-Yam, Israel (~9,000 ka) [1] and an extinct *Bison antiquus* metacarpal from Natural Trap Cave, Wyoming (~17,000 ka) [2]. However, these clear diagnostic data are occasionally overlooked [4], so in this communication the data for these two landmark cases are presented together and reinforced by some new lipid results.

2. Landmark studies

2.1. Nine thousand year old woman and child, Atlit-Yam, Israel

Archaeological investigations off the coast at Atlit-Yam revealed a submerged coastal pre-pottery, post-domestication Neolithic settlement, which included skeletal material from a woman and infant with lesions suggestive of tuberculosis [1,9]. In particular, the inner aspect of the infant cranial bones had serpentine engravings (*serpens endocrania symmetrica*, SES), considered to be diagnostic for intra-thoracic inflammation associated with tuberculosis [1,10]. The tubular bones from the infant, and to a lesser extent from the adult, also demonstrated lesions identified as hypertrophic osteoarthropathy (HOA), highly suggestive of tuberculosis [1,11]. Encouraging results were also achieved for the PCR amplification and sequencing of the *M. tuberculosis* DNA insertion elements IS*6110* and IS*1081*, [1] but additional confirmation was desirable.

Carefully crafted robust lipid biomarker studies were found to be ideal complements to the above compelling evidence for TB in the Atlit-Yam skeletons [1]. The chosen bone samples were degraded by a proven alkaline hydrolysis [1,2,8], designed to release the maximum amount of mycobacterial lipid biomarkers. Quantitative conversion of the acidic fatty components to pentafluorobenzyl (PFB) esters gave lipid biomarkers the ability to be separated reproducibly on silica gel cartridges into fractions containing PFB mycolipenate/mycocerosates, PFB mycolates and free phthiocerols (Figure 1). The latter two lipid classes were converted to stable pyrenebutyric acid (PBA) esters, ideal for sensitive fluorescence HPLC, with the PFB mycocerosate/mycolipenates being amenable to NI–CI GC–MS. The assembled lipid biomarker profiles for the Atlit-Yam specimens are shown in Figure 2.

A simple logical sequence is followed for the HPLC characterisation of mycolic acid derivatives. The initial “reverse phase” HPLC (Figure 2A) serves to isolate and observe any C_{70}–C_{90} mycolates free from any smaller mammalian lipids. A “tight envelope” of total mycolate peaks, as recorded in Figure 2A, is very characteristic for members of the *M. tuberculosis* complex and, indeed, such clear profiles are immediately very positive indications of tuberculosis infections [5,8]. The next stage is to subject the collected total mycolates (Figure 2A) to “normal phase” HPLC to separate the α-, methoxy- and ketomycolate classes (Figure 1A) according to their polarity (Figure 2B); if clear peaks are seen for the individual mycolates, this strengthens the diagnosis of TB. Reverse phase HPLC of each of the collected mycolate types (Figure 2B) can provide very diagnostic profiles (Figure 2C–E). The α-mycolates are relatively homogeneous, a regular series of C_{76} to C_{84} homologues (Figure 2C) having two *cis*-cyclopropane rings (Figure 1A). The oxygenated mycolates, in contrast, comprise two overlapping homologous series with either *cis*- or *trans*-cyclopropane rings (Figure 1A). A very diagnostic feature of the methoxymycolates from *M. tuberculosis* (Figure 2D) is the presence of a double peak comprising the C_{87} *cis*-cyclopropyl and C_{88} *trans*-cyclopropyl methoxymycolates. Similarly, the ketomycolates from *M. tuberculosis* are characteristically dominated by the presence of the C_{87} *trans*-cyclopropyl ketomycolate (Figure 2E). This sequential analysis enables a close correlation to be observed between the mycolate patterns for the Atlit-Yam specimens and standard *M. tuberculosis*.

To support the positive mycolate results, the mycocerosate/mycolipenate (Figure 1B) and phthiocerol family (Figure 1C) profiles have been prepared, in unpublished studies, using NI–CI GC–MS of PFB esters for the former (Figure 2F–I) and fluorescence HPLC of PBA esters for the latter (Figure 2J). Again, the mycocerosate/mycolipenate traces (Figure 2F–I) correspond well with those for standard *M. tuberculosis*. Interestingly, the infant showed