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# The Interplay of DNA and Lipid Biomarkers in the Detection of Tuberculosis and Leprosy in Mummies and other Skeletal Remains

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**Abstract**

The detection of ancient DNA and lipid biomarkers has become established for the detection of tuberculosis and leprosy in archaeological material, including mummies. Recording the characteristic profiles of long-chain mycolic acids by fluorescence high-performance liquid chromatography (HPLC) has been developed to provide a highly specific method for the detection of ancient tuberculosis and leprosy. Initially, mycolate methylanthryl esters were analysed but these derivatives have been superseded by pyrenebutyric acid derivatives of pentafluorobenzyl esters. Long-chain compounds are released by an efficient non-aqueous alkaline extraction and acidic components are converted to stable pentafluorobenzyl esters, which can be preserved for immediate or future analysis. These long-chain components are fractionated into non-hydroxylated esters, mycolates and characteristic phthiocerols. The mycolate pyrenebutyrates are analyzed by fluorescence HPLC to produce profiles characteristic of mycobacterial disease. It is shown in this study that residual material from DNA determinations, on mainly Turkish and Hungarian skeletons, can be used for the detection of mycolic acid biomarkers for tuberculosis and leprosy. The correlation between DNA and mycolic acids biomarker results was not precise, confirming the importance of using complementary methods. In one particular Turkish skeleton, with poor DNA preservation, mycolic acid analysis supported pathological changes indicative of leprosy.

## Introduction

It has been shown that molecular biomarkers, such as DNA and characteristic lipids have good potential in systematic studies of ancient diseases, such as tuberculosis and leprosy (Donoghue 2009). The archaeological material investigated includes skeletons and mummies (Donoghue et al. 1998, 2010; Hershkovitz et al. 2008). The analysis of ancient DNA has allowed the detection and spoligotyping of *Mycobacterium tuberculosis* in Egyptian mummies (Zink et al. 2003).

The analysis of a single biomarker, such as DNA, has limitations due to possible degradation and contamination. The use of stable lipid biomarkers has particular potential for the detection of ancient mycobacterial disease, since high molecular weight mycolic acids, for example, are totally distinct from anything found in mammalian tissue. Modern analytical methods are able to detect such lipids down to the femtomole level and the lack of any amplification reduces the contamination problem. Mycolic acid analysis, using high performance liquid chromatography (HPLC) of fluorescent derivatives, has been shown to be an excellent way to complement DNA detection in recognizing ancient tuberculosis (Donoghue et al. 1998, 2010; Gernaey et al. 2001; Hershkovitz et al. 2008). The mycocerosic acid components of phthiocerol dimycocerosates can also be used to detect *M. tuberculosis* in skeletal material (Redman et al. 2009).

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Archaeological samples have high intrinsic value and it is important to make best use of such material. The present communication will show that it is possible to obtain mycolic acid lipid biomarkers from material from which DNA has already been extracted. The previous protocols, used to detect ancient *M. tuberculosis* mycolic acids for the first time (Donoghue et al. 1998; Gernaey et al. 1998, 2001), suffered from some instability of the chemical derivatives used so a whole new approach has been devised. Mycolic acids are analyzed by fluorescence HPLC of stable pyrenebutyric acid (PBA) derivatives of pentafluorobenzyl (PFB) esters (Donoghue et al. 2010, Hershkovitz et al. 2008, Taylor et al. 2009). This procedure has been used, in combination with DNA analysis, to confirm the presence of tuberculosis in a 9000 year old mother and child from the Eastern Mediterranean (Hershkovitz et al. 2008) and the so-called Granville mummy, preserved in the British Museum (Donoghue et al. 2010). The presence of leprosy was confirmed in an Uzbek leper (Taylor et al. 2009). The intrinsic value of archaeological material requires that information must be maximised and there appeared to be no reason why residues from DNA extraction should not be investigated for the presence of lipid biomarkers, such as mycolic acids.

## Methods

The archaeological materials used (Table 1) were extracts available from previous studies (Donoghue et al. 2005, Molnár et al. 2006, Csóri et al. 2009, Marcsik et al. 2009, Donoghue, unpublished), in which DNA had been removed according to standard protocols (Donoghue et al. 2005). These extracts and standard *M. tuberculosis* and *M. leprae* were processed, as described previously (Donoghue et al. 2010, Hershkovitz et al. 2008, Taylor et al. 2009), to produce extracts suitable for HPLC analysis of mycolates as their PBA-PFB esters. HPLC separations were performed with a VWR Hitachi Elite LaChrom HPLC with an L-2130 pump and L-2480 fluorescence detector. Samples were dissolved in HPLC grade heptane (20−40 μl) in a 1.0 ml vial and-injected by an autosampler L-2200 (0.1−20 μl). The column was stabilised at 25 °C in an L-2300 column oven. Fractions were automatically collected on a Foxy Jr. fraction collector. Conditions for the HPLC analysis are shown in Table 2. Reverse phase columns were used for identification of total mycolates and mycolate subclasses after the total mycolates were collected and separated into α-, methoxy- and ketomycolate subclasses on normal phase columns.

## Results and Discussion

The initial reverse phase HPLC profiles are shown in Figure 1a. The total mycolate fractions were collected and analysed by normal phase HPLC, as shown in Figure 1b the relative proportions of each mycolate class are shown in Table 1. Fractions corresponding to α-, methoxy- and ketomycolates were subjected to further reverse phase HPLC as shown in Figure 2. HPLC profiles of mycolates from standard *M. tuberculosis* and *M. leprae* (Figs. 1, 2) are distinct but there is some overlap of components. The main distinguishing feature is the lack of methoxymycolates in *M. leprae* (Fig. 1b) but other notable differences are, for *M. tuberculosis* and *M. leprae,* the main ketomycolates have 87 and 83 carbons (Fig. 2c), respectively, and the α-mycolates have 80 and 78 carbons (Fig. 2a), respectively.

The total mycolate reverse phase traces (Fig. 1a) included those (samples 4, 7 and 8) which resembled the leprosy standard. In the normal phase profiles of these samples (Fig. 1b, Table 1) there are small components corresponding to ketomycolates and in the region expected for methoxymycolates. Second reverse phase HPLC of the mycolate classes, from samples 4, 7 and 8, showed nothing attributable to methoxymycolates (Fig. 2b); samples 4 and 7 appeared to have ketomycolates similar to *M. leprae* but the extract from sample 8 was devoid of ketomycolates (Fig. 2c). Sample 9 produced an α-mycolate HPLC trace (Fig. 2a), resembling *M. leprae,* but the trace for the ketomycolates was inconclusive with only a very small peak corresponding to a $C_{83}$ component (Fig. 2c). It is important to note, as indicated above, that the presence of a quantifiable amount of material (Table 1) in the region expected for methoxymycolates on normal phase HPLC (marked with "↓" in Figure 1b) does rule out the diagnosis of leprosy. For example, samples 4, 7, 8 and 9 showed small proportions (0.12−0.39, Table 1) of indistinct materials (Fig. 1b), which are not confirmed as methoxymycolates on reverse phase HPLC (Fig. 2b).

The suggestion that mycolate analysis showed that sample 4 was a case of leprosy is particularly interesting, as the poor quality of the aDNA did not allow a diagnosis to be made using that biomarker (Table 1; Donoghue, unpublished). This skeleton (KK24/1a) was obtained from the same Turkish Byzantine

source (Erdal 2004) as another sample (KK20/1), which gave a strong aDNA response for leprosy (Monod et al. 2009). Both of these skeletons showed comparable clear pathology, indicative of leprosy (Erdal 2004), so aDNA preservation can vary within a particular site.

Evidence for the presence of TB was seen in samples 1, 2, 3 and 11, but some of the traces had unusual features (Figs. 1, 2). The mycolates from samples 2 and 11 gave normal phase HPLC traces with double peaks for each of the α-, methoxy- and ketomycolate types (Fig. 1b). This indicates that a type of degradation, called "racemisation", has taken place to produce "diastereoisomers", which are separable on normal phase chromatography. In the second reverse phase profiles (Fig. 2) peaks indicating α-, methoxy- and ketomycolates could be seen, but extra peaks (labeled ?) were observed in α-mycolates from sample 11 and methoxy- and ketomycolates for sample 3. The extra peaks eluted before those attributable to *M. tuberculosis* and they may be due to partial degradation of the oxygenated mycolates.

In the samples 6 and 10 the mycolate HPLC profiles show evidence for co-infection with TB and leprosy. Notably, these extracts are relatively rich in ketomycolates (Fig. 1b, Table 1); that from sample

**Table 1.** Origin and results for archaeological samples. The ratios of individual mycolate types were measured by automatic integration; a number for methoxy-MA does not confirm its presence and the HPLC traces in Figures 1 and 2 must be consulted. The references marked with * contain no aDNA results; the quoted results are Donoghue, unpublished. Ancient leprosy DNA, has been found in a skeleton (KK20/1) from the same site as Sample 4 (KK24/1a) (Monod et al. 2009).

| No. | Date, place and code | aDNA | MA | α-, methoxy-keto-MA ratio | Correlation | Reference |
|---|---|---|---|---|---|---|
| 1 | 7th–8th century Kiskundorozsma, Hungary KD 517 | Lep+TB | TB | 1:0.44:0.33 | +/– | Molnár et al. 2006 |
| 2 | 7th–8th century Kiskundorozsma, Hungary KD 21 | TB | TB | 1:0.74:0.28 | + | Molnár et al. 2006 |
| 3 | 8th–9th century Byzantine, Turkey KK2/1a | TB? | TB | 1:0.52:0.25 | + | Erdal 2004* |
| 4 | 8th–9th century Byzantine, Turkey KK24/1a | Poor DNA ? | Lep | 1:0.15:0.30 | – | Erdal 2004* |
| 5 | 15th century Szombathely, Hungary 6 | TB | ? | 1:0.87:0.10 | – | Donoghue et al. 2005 |
| 6 | 15th century Szombathely, Hungary 10 | Lep | Lep+TB | 1:0.42:0.99 | +/– | Donoghue et al. 2005 |
| 7 | 10th–11th century Püspökladány, Hungary 503 | Lep+TB | Lep | 1:0.12:0.19 | +/– | Donoghue et al. 2005 |
| 8 | Late 7th–9th century Szarvas Grexla, Hungary SG38 | Lep | Lep? | 1:0.13:0.21 | + | Marcsik et al. 2009 |
| 9 | 10th century Hajdudorog-Gyulas, Hungary HG56 | Lep | Lep? | 1:0.39:0.09 | + | Csóri, et al. 2009 |
| 10 | 8th century Kiskundorozsma, Hungary KD9018 | Lep | Lep+TB | 1:1.00:1.46 | +/– | Molnár et al. 2006 |
| 11 | 19th century Crossbones, Southwark, London, UK 9 | TB | TB? | 1:0.88:0.58 | + | Brickley et al. 1999* |
| 12 | 10th–13th century Bjorned, Sweden A4 | Lep+TB | ? | 1:0.87:0.30 | – | Donoghue et al. 2005 |

**Table 2.** Conditions for HPLC analysis of PBA PFB mycolates.

| Conditions | Column | Gradient elution program |
|---|---|---|
| Flow rate: 1 ml/min. | Reverse phase: Alltech Alltima 81412 C18 analytical column, 3 µ, I.D. 4.6 mm × Length 50 mm | Acetonitrile/tetrahydrofuran: 55:45 to 45:55 in 31 min |
| Detector: Excitation 342 nm, emission 376 nm | Normal phase: Alltech Alltima 81414 silica analytical column, 3 µ, I.D. 4.6 mm × Length 50 mm | Heptane/ethyl acetate: 100:0 to 99:1 in 1 min; 99:1 to 97:3 in 30 min |

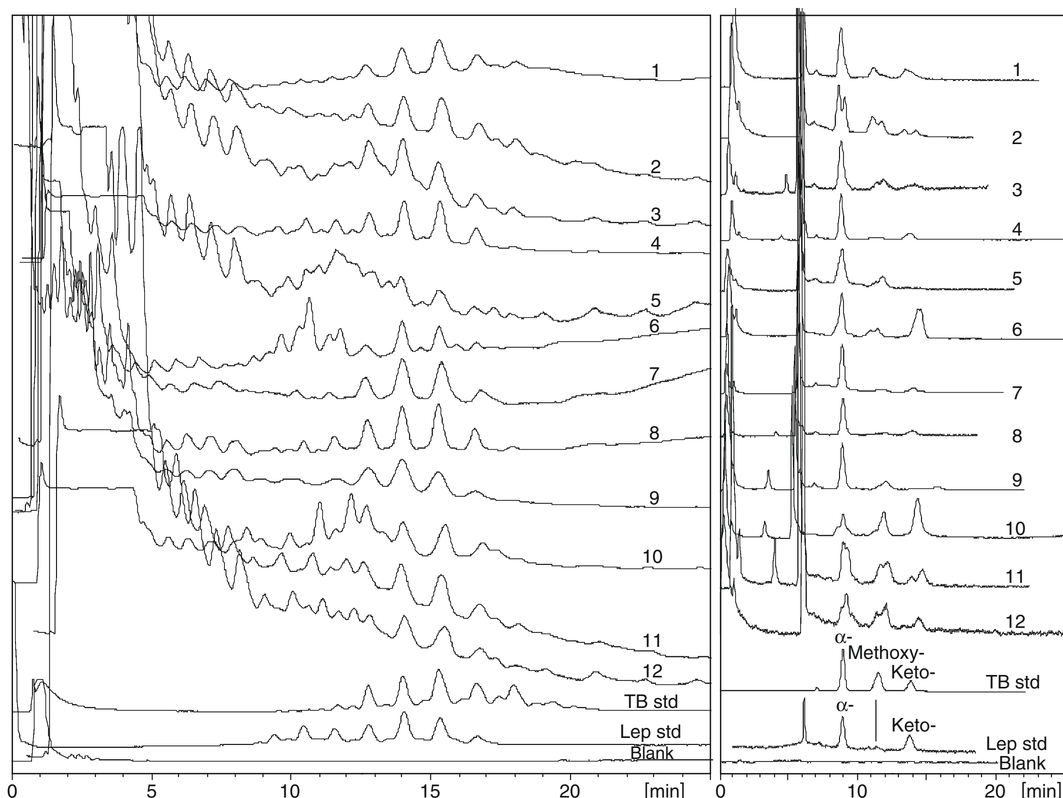


**Fig. 1.** HPLC of PBA-PFB derivatives of MAs. **a,** Reverse phase HPLC of total mycolates. **b,** Normal phase HPLC of collected total mycolate fraction. See Table 1 for origin of samples 1 to 12. "TB std" and "Lep std" are standard mycolic acid profiles for *M. tuberculosis* and *M. leprae,* respectively. The arrow ("↓") in Figure 1b indicates the absence of methoxymycolates in *M. leprae.*

6 corresponds well in size with that ($C_{83}$) of *M. leprae* but the ketomycolate from sample 10 appears to contain both $C_{83}$ and $C_{85}$ components. Both samples 6 and 10 had recognizable methoxymycolate HPLC traces (Fig. 2b), supporting the presence of *M. tuberculosis*; the main α-mycolates had main $C_{78}$ components, favoring *M. leprae* (Fig. 2a). Extracts of samples 5 and 12 have poorly defined profiles, which indicate a substantial degree of degradation, particularly for sample 5.

It is interesting to correlate the above results with previous studies of ancient DNA (aDNA) (Table 1). Good correlation is seen with samples 2, 3, 8, 9 and 11, with 2, 3 and 11 confirming TB and 8 and 9 supporting leprosy. Interesting disparities were observed for samples 1, 6, 7 and 10. For sample 1, both TB and leprosy were indicated by aDNA analysis, but the HPLC profiles (Figs. 1, 2) were in favor of only TB. Similarly, aDNA showed both diseases in sample 7, but lipid analysis suggested only leprosy. Conversely, samples 6 and 10 had only recognizable DNA for *M. leprae* but both diseases were discernible from the mycolate HPLC profiles (Figs. 1, 2). In sample 4, which had strong leprosy pathology, aDNA studies were inconclusive (Table 1), but reasonably clear HPLC mycolate profiles were recorded to indicate leprosy. Conversely, where conclusive aDNA results suggested TB and TB plus leprosy for samples 5 and 12, respectively (Table 1), the mycolate HPLC profiles (Figs. 1, 2) were uninformative.

The results summarized in Figures 1 and 2 demonstrate that residues remaining after DNA extraction are still a source of mycolic acid biomarkers for mycobacterial disease. This is not surprising as the aDNA extraction protocols are completely aqueous and mycolic acids are extremely hydrophobic. The recorded profiles do not all correlate precisely with standard material from *M. tuberculosis* and *M. leprae* (Figs. 1, 2). In previous studies on *M. tuberculosis* (Donoghue et al. 2010; Hershkovitz et al. 2008; Gernaey

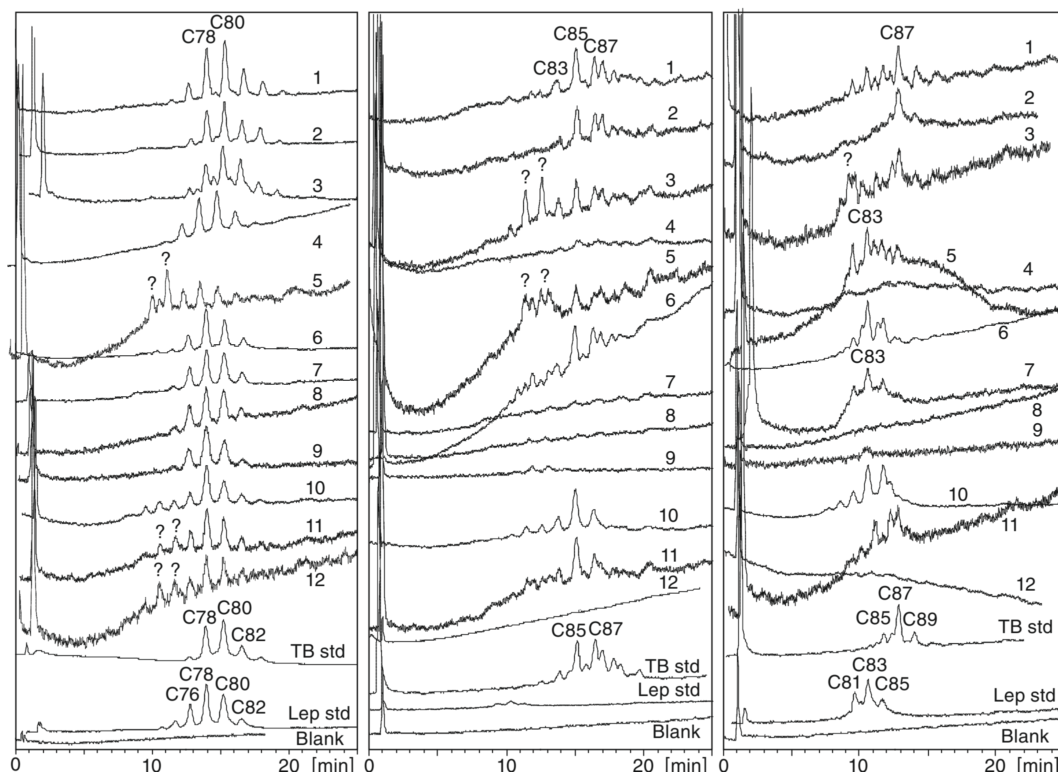


**Fig. 2.** Reverse phase HPLC of collected PBA-PFB derivatives of MAs. **a,** α-Mycolate fraction. **b,** Methoxy-mycolate fraction. **c,** Ketomycolate fraction. See Table 1 for origin of samples 1 to 12. "TB std" and "Lep std" are standard mycolic acid profiles for *M. tuberculosis* and *M. leprae*, respectively. Unusual peaks are indicated by "?".

et al. 1998, 2001), it appeared that the mycolic acid content remained essentially undegraded, indicating that preservation of those samples was good. The HPLC results, shown here (Figs. 1, 2), are not so pristine, suggesting various levels of degradation. It is not possible to conclude, from the present study, that mycolic acid lipid biomarkers are more robust than aDNA. The main finding is that the joint analysis of both aDNA and mycolate biomarkers, on the same sample, provides an enhanced level of diagnostic information.

In exploiting the use of molecular biomarkers for ancient mycobacterial disease, it is important to ensure that a rigorous and objective approach is taken. In a recent critique of ancient DNA studies (Wilbur et al. 2010), a number of concerns were expressed and a detailed response has been published to allay such fears (Donoghue et al. 2009). Similarly, it has been claimed that characteristic mycolic acids can be detected in a high through-put mass spectrometric method for the detection of ancient tuberculosis (Mark et al. 2010). However, the recorded profiles did not correspond to previously recorded total *M. tuberculosis* mycolic acid profiles (Laval et al. 2001), on which the procedure was based. Indeed, analysis of commercial standard mycolic acids did not even produce a recognizable profile. It cannot be concluded that a positive diagnosis of ancient tuberculosis was achieved by Mark et al. (2010).

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