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Analysis of ancient mycolic acids by using MALDI TOF MS: response to “Essentials in the use of mycolic acid biomarkers for tuberculosis detection” by Minnikin et al., 2010

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The mycobacterial cell wall is consists of several long chain lipid and glycolipid components. Mycolic acids are major and unique fraction of the cell envelope of mycobacteria that include the ancient causative agents of tuberculosis and leprosy. The analytical investigation of these lipid biomarker residues is one of the most promising perspectives in the field of molecular paleopathology. Recently published in this journal, Minnikin et al. present a systematic critique of the MALDI TOF MS analysis for determination of ancient mycolic acids, focusing on our previous paper. In this study, our mass spectrometric investigations by commonly used 2,5 DHB matrix were presented with our comments to the critique authors. On the base of our previous and recent mass spectrometric results we have to realize that the clinical protocols and standards cannot directly be used for the biomolecular paleopathological investigations. The applicability of the recent mycobacterial and clinical results is very limited in the biomolecular archaeology, thus the recent scientific results and protocols should be adapted carefully to bioarchaeological sciences.

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1. Introduction

It is a widely accepted view that diagnosis of tuberculosis from archaeological human skeletal remains is not an easy task by using classic morphological methods. A biomolecular approach to diagnosis is probably more reliable than gross osteological examination of archaeological skeletal remains (Donoghue et al., 2004, 2009; Donoghue, 2009; Stone et al., 2009; Wilbur et al., 2009). The structure of the recent mycobacterial cell envelope is well known, contains a characteristic mycoloyl arabinogalactan–peptidoglycan complex. Mycolic acids, as extremely hydrophobic, long chain fatty acid residues of mycobacterial cell envelope, are ideal targets for biomolecular detection of ancient tuberculosis.

Recently published in this journal, Minnikin et al. (2010) present a systematic critique of the matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI TOF MS) analysis for determination of ancient mycolic acids, focusing on our previous paper (Mark et al., 2010). The critique paper contains no original mass spectrometric data from archaeological skeletal remains, only MALDI TOF MS spectra of recent mycobacterial mycolic acids have been presented. The mass spectrometric analysis of total mycolic acids, total methyl mycolates, methyl α-mycolates, methyl methoxymycolates and methyl ketomycolates were carried out by using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix (Minnikin et al., 2010, Fig. 2), further mass spectrometric properties were not mentioned in the paper.

2. Materials and methods

2.1. Mycolic acid standards and archaeological remains

In this study a mycolic acid standard (Mycolic acid from *Mycobacterium tuberculosis* human strain, CAS 37281-34-8, Cat. No. M4537) from Sigma–Aldrich Kft. (Budapest, Hungary) and mycolic acid standards from David E. Minnikin as well as tuberculosis infected and non-infected archaeological skeletal remains have been analyzed. The measured archaeological bone samples have been summarized in Table 1.

2.2. MALDI TOF mass spectrometry

The sample preparation and mass spectrometric analysis were carried out as previously described in Mark et al. (2010) with few

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Table 1
Anthropological data of the presented archaeological skeletal remains (MT – *Mycobacterium tuberculosis* infected archaeological bone samples).

Location	Period	Grave	Age	Sex	Pathology
Sükösd-Ságod	600–700 A.D.	19	19–20	Female	MT
		195	30–40	Female	Control
Bélmegyer-Csömöki domb	700–800 A.D.	65	30–40	Female	MT
		68	40–50	Male	Control
Felgyő-Ürmös tanya	700–800 A.D.	205	20–30	Female	MT
		232	40–50	Female	Control
Csongrád-Ellés	1000–1200 A.D.	183	40–45	Male	MT
		95	40–50	Male	Control

modifications. Briefly, the bone samples (thoracic vertebra) were washed with tap water and dried at room temperature; the powder was ground by hand with an agate mortar, the particle size was ca. 0.2 mm. 100.0 mg of bone powder was extracted with 200 μL chloroform (Sigma–Aldrich Kft., Budapest, Hungary) – methanol (Sigma–Aldrich Kft., Budapest, Hungary) (80/20 v/v) mixture in an ultrasonic bath at 10 min. The extracts of the non-infected and the tuberculosis infected ancient bones as well as the 0.1 mg $\times$ mL^{-1} solutions of mycolic acid standards were loaded onto the target plate (MTP 384 massive target T, Bruker Daltonics, Bremen, Germany) by mixing 1.0 μL of each solution with the same volume of a matrix solution, prepared by dissolving 10 mg of 2,5-dihydroxybenzoic acid in chloroform (Sigma–Aldrich Kft., Budapest, Hungary) – methanol (Sigma–Aldrich Kft., Budapest, Hungary) (80/20 v/v) mixture. The mass spectrometer used in this work was an Autoflex II TOF/TOF (Bruker Daltonics, Bremen, Germany) operated in the reflector detector. An accelerating voltage of 20.0 kV was used for analyses. The instrument uses a 337 nm pulsed nitrogen laser, model MNL-205MC (LTB Lasertechnik Berlin GmbH., Berlin, Germany). External calibration was performed in each case using Bruker Peptide Calibration Standard (#206195 Peptide Calibration Standard, Bruker Daltonics, Bremen, Germany). Masses were acquired with a range of m/z 700–m/z 3000. Each spectrum was produced by accumulating data from 500 consecutive laser shots. The Bruker Flex Control 2.4 software (Bruker Daltonics, Bremen, Germany) was used for control of the instrument and the Bruker Flex Analysis 2.4 software (Bruker Daltonics, Bremen, Germany) for spectra evaluation.

2.3. NMR analysis

^{1}H NMR spectra of the mycolic acid standard from Sigma–Aldrich were recorded with a Varian Unity Inova 400 WB spectrometer (Varian Inc., Palo Alto, CA, USA).

3. MALDI TOF mass spectrometry in detection of ancient tuberculosis

In our previous paper (Mark et al., 2010) a high-throughput mass spectrometric technique and a Fourier transform infrared spectroscopic method were mentioned for distinguishing of *M. tuberculosis* infected and non-infected ancient skeletal remains (Mark et al., 2010, Fig. 1, Figs. 3–4 and Fig. 5). The main aim of our study was to present a novel strategy for determination of ancient tuberculosis infections and not the accurate annotation of the mass spectrometric peaks and the corresponding analytes. Forasmuch, the mycolic acids and their derivatives (eg. trehalose monomycolates, trehalose dimycolates etc.) have a complex structure and their "chemical fossilization" is completely unknown. The annotation of each mass spectrometric peak and the identification of the chemical structure of the ancient tuberculosis biomarkers can be possible in the view of the physico-chemical diagenesis of the mycolic acids during the period of hundreds or thousands of years. In our opinion, it would be very surprising if the mass spectra of ancient mycolic acid biomarkers were exactly the same as the those of recent standards and clinical samples. It is well known that the mycolic acids are relatively stable molecules, but numerous chemical reactions can be possible during the time such as redox reactions, amidation, carbamidomethylation, ester formation, saponification, fragmentation, enzymatic hydrolysis, enzymatic condensation, opening of the cyclopropane ring, methylation etc. Accordingly, the annotation of the mass spectrometric peaks and the identification of ancient mycolic acids and their metabolites could only be carried out with accurate and systematic chemical modeling of the mycolic acid postmortem diagenesis. Furthermore, the fatty acid biosynthesis is an essential part of *M. tuberculosis* lifestyle and evolution with the genome containing ca. 250 fatty acid metabolizing enzymes. This pathway has been extensively modified during the adaptive evolution of the bacteria (Kinsella et al., 2003). Accordingly, not only postmortem changes are feasible, but the synthesis of unique mycolic acids could be possible in vivo, during the lifetime of ancient *Mycobacterium*. The sensitive and specific determination of the suggested chemical modifications and structural changes of ancient mycolic acids can only be possible by using modern tandem mass spectrometric methods. Previously, chromatographic methods were used for analysis of mycolic acids from the archaeological bone samples (Donoghue et al., 2010; Gernaey et al., 2001; Hershkovitz et al., 2008). For separation, both normal- and reversed-phase high-performance liquid chromatography (NP-HPLC, RP-HPLC) are commonly used. Normal-phase chromatography is not a "simple" technique for the accurate separation of the components as Minnikin et al. described in their critique paper. For this method the stationary phase is hydrophilic (usually silica) and the mobile phase is a hydrophobic eluent or eluents such as benzene, toluene etc. It is well known that the reproducibility of the normal-phase separation is very low, because many properties are affected by the adsorption mechanism eg. water content of the mobile phases, temperature, the number and the activity of the free silanol groups on the surface of the stationary phase as well as the ratio of the adsorbed layer volume of the mobile phase and the non-adsorbed mobile phase volume etc. (Neue, 1997). The complete NP-HPLC or RP-HPLC separation of ancient mycolic acids and their derivatives from an extract of an archaeological skeletal remains, especially the identification of the separated ancient biomarkers by using only the retention times (and not using the retention factors or online mass spectrometric detection) is a very hard task. Recently, an outstanding paper of the critique authors has been published about the biomolecular investigations of Dr Granville's mummy (Donoghue et al., 2010). In their study the ancient tuberculosis infection was detectable by using HPLC analysis of mycolic acids. However, several significant differences can be observed on the standard chromatograms and the results of the bone samples. Some unexplainable major and minor peaks were detected in the extracts of the archaeological remains in contrast to *M. tuberculosis* standard (Donoghue et al., 2010, Figs. 4–5; as well as in Redman et al., 2009 Fig. 5). Furthermore, the retention times as well as the retention factors (Donoghue et al., 2010, on Fig. 5a, t_0 of the *M. tuberculosis* standard or on Fig. 5b, t_0 of the lung sample 1) and resolutions were shifted in several cases (Donoghue et al., 2010, Figs. 4 and 5b). One possible explanation of these irregularities would be the chemical modifications of the ancient mycolic acids during the chemical diagenesis.

In Mark et al. (2010), C_{60} fullerene and 2,5-DHB as matrices were used for MALDI TOF analysis and only the results with fullerene were presented in the paper (Mark et al., 2010, Figs. 2–5) because of the novelty of the method. The analytical applications of fullerenes

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