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Original Article

Impact of storage conditions on metabolite profiles of sputum samples from persons with cystic fibrosis

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Abstract

Background: Although recent studies have begun to elucidate how airway microbial community *structure* relates to lung disease in cystic fibrosis (CF), microbial community *activity* and the host's response to changes in this activity are poorly understood. Metabolomic profiling provides a means to investigate microbial activity and human cell activity within diseased airways. However, variables in sample storage and shipping likely affect downstream analyses and standards for sample handling are lacking.
Methods: We assessed the impact of sample storage conditions on liquid chromatography mass spectrometry analysis of CF sputum samples.
Results: Significant changes in global metabolomic profiles occurred in samples stored at room temperature or at 4 °C for longer than one day. Untargeted metabolomic profiles were stable in sputum samples stored at −20 °C or −80 °C for at least 28 days. Quorum sensing molecules and phenazines, both considered important to the in vivo activity of *Pseudomonas* during airway infection, were detected after sample storage at room temperature for five days.
Conclusions: Sputum samples can be stored at −20 °C or −80 °C for weeks with minimal effect on global metabolomic profiles. This observation provides guidance in designing metabolomic studies that have the potential to deepen our understanding of how airway microbial communities impact lung disease progression in CF.

Keywords: Cystic fibrosis; Metabolome; Sputum

1. Introduction

The use of next generation DNA sequencing is expanding our understanding of respiratory tract infection in persons with cystic fibrosis (CF). Several studies provide compelling evidence that CF airways typically harbor complex bacterial communities — and that changes in the structure of these communities are associated with lung disease progression [1–5]. How changes in microbial community structure may relate to shorter term changes in clinical state (e.g., the onset of pulmonary exacerbation) is less clear. Studies addressing this have observed varying degrees of community structure change [6–10], but have been limited by relatively small numbers of samples and by the technical challenges inherent in microbiome analyses [11,12].

Metabolomic profiling of sputum samples offers a means to assess bacterial *activity* within the airways and to correlate this activity with clinical outcomes. Struss and colleagues [13], for example, recently used liquid chromatography coupled with mass spectrometry (LC–MS) to show that the level of the *Pseudomonas* quorum sensing molecule N-3-oxododecanoyl homoserine lactone (3-oxo-C_{12}-HSL) was higher in CF sputum samples obtained during hospitalization for exacerbation than in samples obtained from 'stable' patients. In addition to

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investigating bacterial metabolites, sputum metabolomic analyses can also detect human metabolites, enabling investigation of bacterial–human interactions and the identification of biomarkers that may be useful in monitoring lung disease progression [14–16].

Despite the potential utility of metabolomic analyses in advancing our understanding of CF pathobiology, there is a paucity of data regarding the appropriate methodology for sputum metabolomic studies. Unlike biological samples collected from animal models under highly controlled conditions, there is potential for considerable variation in the collection, shipping, processing, and storage of sputum samples from CF patients. These factors could very well influence metabolomic analyses, diminishing the likelihood of detecting or accurately quantitating metabolite markers of disease activity and progression. We assessed the stability of CF sputum metabolomic profiles while varying sample storage and handling conditions likely to be encountered in longitudinal outcomes studies. In so doing, we provide guidance to the design of studies aimed at seeking associations between airway community activity and clinical condition.

2. Methods

2.1. Sputum collection and storage

With approval from the University of Michigan Institutional Review Board, expectorated sputum specimens were collected from three adult CF patients receiving care at the University of Michigan Health System. Sample 1 was stored at 4 °C overnight before being processed further. Samples 2 and 3 were processed immediately after collection. Each sample was processed by first being combined with an equal volume of cold sterile water and homogenized mechanically with a sterile tissue homogenizer (Omni International) on ice for 10 s or until uniformly distributed. Each sample was then divided into 50 μL aliquots and duplicate aliquots were stored at different temperatures for various lengths of time before being stored at −80 °C (Fig. 1). This yielded a total of 56 sputum aliquots for metabolomic profiling.

2.2. Metabolite extraction and metabolomic profiling

We chose to use methanol as an extraction solvent since previous studies have found it to be suitable for extracting a wide range of hydrophilic and hydrophobic metabolites from a variety of sample types [17,18]. Further, we elected to use LC–MS as a platform for untargeted metabolomic profiling since it is generally regarded as offering the broadest metabolome coverage of any single analytical strategy [19].

Sputum aliquots were thawed on ice and extracted by the addition of 200 μL of methanol containing the following stable isotope labeled internal standards: 10 μg/mL ^{13}C amino acid mix, 40 μM $^{13}C_3$ lactate, and 2 μM $^{13}C_6$ citrate (all purchased from Sigma-Aldrich). The samples were vortexed for 5 s, then centrifuged at 16,000 ×*g* for 10 min. From each sample, 200 μL of supernatant was recovered and dried using a Speedvac at 60 °C. The residue was reconstituted in 30 μL of 1:1 methanol:water and analyzed by reversed phase LC–MS (RPLC–MS) using an Agilent 1200 LC/6520 quadrupole-time of flight MS system (Agilent Technologies, Santa Clara, CA) operated in full-scan positive ion mode (m/z 50–1200). A Waters Acquity HSS T3 1.8 μ column, 50 mm × 2.1 mm ID (Waters Corporation, Milford, MA) was used to separate metabolites. Mobile phase A was 100% water with 0.1% formic acid and mobile phase B was 100% acetonitrile with 0.1% formic acid. The gradient

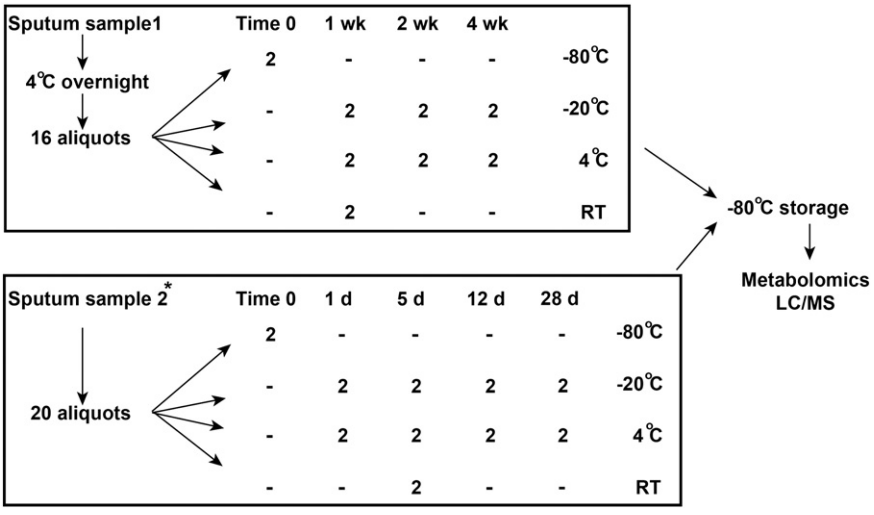

Fig. 1. Experimental design. The sample from the first individual (sputum sample 1) was stored at 4 °C overnight before it was divided into 16 aliquots (50 μL each). Two of the aliquots were stored at −80 °C, while others were stored in duplicate at 4 °C or −20 °C for 1 week, 2 weeks, or 4 weeks. Two aliquots were stored at room temperature for 1 week. The sputum samples from the second and third individuals (sputum sample 2 and sputum sample 3) were each divided into 20 aliquots (50 μL each) immediately after collection. Two aliquots of each sample were stored at −80 °C immediately, while others were stored in duplicate at 4 °C or −20 °C for 1 day, 5 days, 12 days or 28 days. Two aliquots of each sample were stored at room temperature for 5 days. After the specified storage period, all aliquots were stored at −80 °C.