Journal of Cystic Fibrosis 14 (2015) 464–467

www.elsevier.com/locate/jcf

Short Communication

Implications of multiple freeze-thawing on respiratory samples for culture-independent analyses ☆

Leah Cuthbertson [a,b], Geraint B. Rogers [c], Alan W. Walker [d,e], Anna Oliver [a], Lucas R. Hoffman [f,g], Mary P. Carroll [h], Julian Parkhill [d], Kenneth D. Bruce [b], Christopher J. van der Gast [a,*]

[a] NERC Centre for Ecology & Hydrology, Wallingford, OX10 8BB, UK
[b] Institute of Pharmaceutical Science, Molecular Microbiology Research Laboratory, King's College London, London SE1 9NH, UK
[c] SAHMRI Infection and Immunity Theme, Flinders University, Adelaide, Australia
[d] Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, UK
[e] Rowett Institute of Nutrition and Health, Aberdeen AB21 9SB, UK
[f] Department of Pediatrics, University of Washington, Box 356320, HSB K328A, Seattle, WA 98105, USA
[g] Department of Microbiology, University of Washington, Box 356320, HSB K328A, Seattle, WA 98105, USA
[h] Cystic Fibrosis Unit, Southampton University Hospitals NHS Trust, Southampton SO16 6YD, UK

Received 17 July 2014; revised 3 October 2014; accepted 13 October 2014
Available online 24 October 2014

Abstract

Background: Best practice when performing culture-independent microbiological analysis of sputum samples involves their rapid freezing and storage at −80 °C. However, accessing biobanked collections can mean that material has been passed through repeated freeze–thaw cycles. The aim of this study was to determine the impact of these cycles on microbial community profiles.
Methods: Sputum was collected from eight adults with cystic fibrosis, and each sample was subjected to six freeze–thaw cycles. Following each cycle, an aliquot was removed and treated with propidium monoazide (PMA) prior to DNA extraction and 16S rRNA gene pyrosequencing.
Results: The impact of freeze–thaw cycles was greatest on rare members of the microbiota, with variation beyond that detected with within-sample repeat analysis observed after three cycles.
Conclusion: Four or more freeze thaw cycles result in a significant distortion of microbiota profiles from CF sputum.

Keywords: Microbiota; Microbiome; Biobank; Pyrosequencing; Propidium monoazide; Sputum

1. Introduction

The application of next-generation sequencing technologies for the investigation of lower respiratory tract infections in patients with cystic fibrosis has revealed complex and highly diverse microbial communities [1,2]. As technologies have improved and the associated costs have fallen, it is becoming possible to use these platforms, not only for research but also for diagnostic microbiology [3], making it more important than ever to identify and minimise the introduction of bias.

Spontaneously expectorated sputum is one of the most common specimen types used to investigate the microbial community responsible for lower respiratory infections in adults with CF. In order to perform culture-independent analysis on a representative airway sample, the methods used to collect and store specimens are hugely important. Current best practice involves the rapid stabilisation of sputum samples by freezing at −80 °C within 12 h of collection [4]. To allow

Abbreviations: PMA, Propidium monoazide; OTU, Operational taxonomic units; *S**, Species richness; *H′*, Shannon–Wiener; 1-*D*, Simpson's; S_{BC}, Bray–Curtis.

☆ Data deposition: The sequence data reported in this paper have been deposited in the NCBI Short Read Archive database (Accession number SRP040968).

* Corresponding author.
E-mail address: cjvdg@ceh.ac.uk (C.J. van der Gast).

http://dx.doi.org/10.1016/j.jcf.2014.10.004

profiling of the viable microbial community, samples can be treated with propidium monoazide (PMA) to remove the impact of extracellular DNA or DNA from dead and damaged cells, prior to DNA extraction and DNA sequencing [5]. Received wisdom suggests that once defrosted, respiratory samples may not be refrozen and sub-sampled again at a later date without incurring significant changes in the microbial community.

There is an increasing awareness of the importance of in-depth analysis for the investigation of the microbial communities responsible for infection. This has lead to many clinics collecting large detailed sample biobanks in ultralow temperature freezers, which can be accessed in order to address a wide range of clinical questions. However, biobanked samples may be accessed multiple times for culture independent analysis, thus passing though several freeze thaw cycles, a process that could result in changes to the microbial community. To date, no studies have used next-generation sequencing technologies to define how multiple freeze–thaw cycles affect the microbial community within collected sputum. We hypothesized that microbial community profiles would be significantly altered with each additional freeze–thaw cycle when analysed using 16S rRNA gene pyrosequencing.

2. Methods

2.1. Sample collection

Sputum samples were collected, under full ethical approval from the Southampton and South West Hampshire Research Ethics Committee (06/Q1704/26), from eight patients attending the regional Cystic Fibrosis Centre in Southampton General Hospital. All patients were chronically colonised with *Pseudomonas aeruginosa*. Patients were selected based on their ability to typically produce more than 2 ml of sputum. Sputum samples were collected and frozen at −80 °C within 1 h. Each sputum sample was subjected to six freeze–thaw cycles. Samples were removed from the −80 °C freezer, a 250 μl aliquot removed for DNA extraction, and the remaining sample allowed to completely thaw at room temperature for 30 min before being returned to −80 °C for 24 h.

2.2. DNA extraction and pyrosequencing

Sputum samples were washed three times with 1× phosphate buffered saline to remove saliva, as previously described [1]. Extracellular DNA and DNA from non-viable cells were excluded from analysis via crosslinking with PMA [6,7] prior to DNA extraction, as described previously [8]. Bacterial Golay barcode-encoded FLX amplicon pyrosequencing was performed using the primer 338F (3′- ACTCCTACGGGAGGCAGCAG) and 926R (3′- CCGTCAATTCMTTTRAGT). Initial generation of 16S rRNA gene amplicons involved a one step PCR of 25 cycles using AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA). 454 pyrosequencing using the Lib-L kit was performed at the Wellcome Trust Sanger Institute, Hinxton, UK.

Resulting data were analysed using the Mothur sequencing analysis platform [9] as described previously [4]. The raw sequence data generated within the current study have been submitted to the NCBI Short Read Archive database under the study accession number SRP040968. The barcodes associated with each sample are shown in Table S1. Two aliquots were excluded due to insufficient number of sequence reads generated.

2.3. Statistical analysis

Statistical analysis was performed in R [10]. Changes in bacterial diversity were assessed using three complementary measures: species richness (S*, the total number of species), Shannon–Wiener (H', a metric accounting for both number and relative abundance of species), and Simpson's (1-D, a measure of the probability that two species randomly selected from a sample will differ) indices of diversity as described previously [4,11]. The Bray–Curtis (S_{BC}, which accounts for the number and abundance of species present in each community and those that are shared), resulting in a value between 0 and 1 (higher values indicating greater similarity) measure of similarity was used to assess changes in community composition with each freeze–thaw cycle.

To avoid potential bias, all measures were calculated using randomised resampling to a uniform number of sequence reads per sample [5]. Mean diversity measures were calculated from the re-sampling of the reads from each specimen to the lowest number of sequence reads among all specimens (n = 261) for 1000 iterations. S_{BC} was calculated by re-sampling to the minimum number of sequence reads per specimen within each patient and comparing community composition to the original sample for 1000 iterations. Bacterial species detected at the first point for each patient were partitioned into common and rare species using rank abundance curves [12]. The R package nlme [13] was used to fit mixed effect models to investigate the relationships between measures of diversity, similarity, and number of freeze thaw cycles. r^2 values were calculated using the MuMIn package [14].

3. Results

To test the study hypothesis, sputum samples from eight CF patients were subjected to six freeze–thaw cycles. Aliquots of sputum were removed for DNA extraction and 16S rRNA gene pyrosequencing to assess the bacterial community after each cycle, and the remaining sample was allowed to defrost completely before being returned to −80 °C. All samples were treated with PMA prior to DNA extraction to focus the analysis on the viable bacterial community. A total of 106,065 sequences (mean ± standard error (SE) per sample 2306 ± 239) were generated from 46 samples, identifying 49 genera and 76 distinct operational taxonomic units (OTUs) classified to species level (Table S2).

3.1. Bacterial diversity

Species richness, S*, was found to be highly variable between patients (max = 49, min = 4), no pattern in raw richness values