Online supplement for

Protected sampling is preferable in bronchoscopic studies of the airways microbiome

MATERIAL AND METHODS

Study design

The design of the MicroCOPD study has been published previously (1). The current analysis includes 58 control subjects, 64 subjects with COPD, and 3 subjects with asthma, all examined between 11th of April, 2013 and 14th of April, 2015 at the outpatient clinic of the Department of Thoracic Medicine at Haukeland University Hospital. All participants were at least 35 years old, and were recruited from previous longitudinal case-control studies (2), and a few volunteers. Participation was postponed for subjects who had an ongoing respiratory symptom exacerbation or had used antibiotics or oral corticosteroids within the last 14 days. Subjects using anti-coagulants or double platelet inhibition, subjects with unstable coronary heart disease, hypoxemia (SpO2 < 90% when receiving 3 liters of oxygen/minute through a nasal canula), hypercapnia at rest (pCO2 > 6.65 kPa), or with known allergies against lidocaine or alfentanil were not included.

Control subjects had a post-bronchodilator (BD) FEV_1/FVC ratio ≥ 0.70 and no clinical diagnosis of obstructive lung disease as evaluated by the study physician. COPD cases had a post-BD FEV_1/FVC < 0.70, and a clinical diagnosis of COPD. Moderate, severe and very severe COPD was defined as post-BD FEV_1 between 50 and 80% of predicted, < 50% of predicted and < 30% of predicted by Norwegian pre-BD reference values,

respectively (3). The Regional Committee for Medical and Health Research Ethics approved the study (REK Nord, project number 2011/1307). All participants provided written informed consent.

Sample collection

All participants received at least 0.4 mg of salbutamol through a spacer before the bronchoscopy procedure. Sterile phosphate-buffered saline (PBS) in bottles of 500 mL were unsealed maximum 24 hours before the procedure. Immediately before bronchoscopy all participants delivered an oral wash (OW) sample by gargling 10 mL PBS. 1 mL of PBS from the same bottle was used as a negative control sample, and all PBS fluid used for samples for one subject came from the same bottle.

Flexible video-bronchoscopy was performed via the oral route in supine position. No suction was used prior to having entered the trachea. All subjects received local anesthesia with lidocaine both before and during the procedure. All but 18 subjects received mild sedation alfentanil parenterally. Participants were monitored according to current guidelines, and were observed for at least 2 hours after the procedure (4).

The following samples were taken in the same consecutive order during bronchoscopy:

1. Three wax-plug protected specimen brushes (PSB) from the right lower lobe (Conmed, Utica, NY, USA). The three brushes were cut off with sterile scissors, and placed together in an Eppendorf tube with 1 ml PBS.
2. Protected bronchoalveolar lavage (PBAL) of the right middle lobe by instilling two fractions each of 50 mL PBS (PBAL1 and PBAL2) using a wax-plug protected catheter (Plastimed Combicath, Le Plessis Bouchard, France).

3. Three wax-plug protected specimen brushes (PSB) from the left upper lobe, treated as the right lobe PSBs.
4. Small-volume lavage (SVL) of 20 mL PBS in the left upper lobe, from the same segment as the left PSB was taken. This lavage was sampled using the suction from the broncoscope´s working channel, thus mimicking the way BAL is most often sampled.

For 49 participants we examined the left side before the right (i.e according to the numbering above we performed sample 3., 4., 1., 2.).

DNA Extraction

1800 µl of OW, PBAL and SVL samples and 450 µl PSB and PBS NC samples were used for DNA extraction. An equal volume of Sputasol (Oxoid) was added to the samples followed by a 15 minute incubation in a thermomixer (1000 rpm) at 37 °C. The bacterial cells were then pelleted by centrifugation at 15700 g for 8 minutes. The supernatant was discarded and the bacterial cells were resuspended in 250 µl PBS.
Bacterial DNA was then extracted from the cells using enzymatic and mechanical lysis methods. As mechanical lysis methods tend to result in the shearing of free DNA, the samples were first treated with an enzyme cocktail consisting of 25 µl lysozyme (10 mg/mL, Sigma-Aldrich), 3 µl mutanolysin (25 KU/mL, Sigma-Aldrich), 1.5 µl lysostaphin (4000 U/mL, Sigma-Aldrich) and 20.5 µl TE5 buffer (10 mM Tris-HCl, 5mM EDTA, pH 8) and incubated at 37°C for 1 hour in a thermomixer (350 rpm). Before proceeding with mechanical cell lysis, the samples were centrifuged at 15700 g for 15 minutes to pellet any bacterial cells not sufficiently lysed by the enzymes. The supernatant containing the extracted DNA was transferred to a new eppendorf tube and stored at 4 °C while further processing of the bacterial cell pellet. The pellet was resuspended in 800 µl CLS-TC lysis

buffer from the FastDNA Spin Kit (MP Biomedicals, LLC, Solon, OH, USA) and transferred to a Lysing Matrix A tube (FastDNA Spin Kit). The sample was then subjected to mechanical lysis using the FastPrep-24 instrument (MP Biomedicals) at a speed setting of 6.0 m/s for 40 seconds. The lysate was then pooled with the supernatant from the enzyme lysis step and DNA further purified as described by the manufacturers for the FastDNA Spin Kit.

16S rRNA library preparation and high-throughput sequencing

Library preparation and sequencing of the V3-V4 region of the 16S rRNA gene was carried out according to the Illumina 16S Metagenomic Sequencing Library Preparation guide (Part # 15044223 Rev. B). The V3-V4 region was PCR amplified and prepared for a subsequent index PCR step using primers adapted from Klindworth et al. (5):

- 16S Amplicon PCR Forward Primer (overhang adaptor sequences underlined) = 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
- 16S Amplicon PCR Reverse Primer (overhang adaptor sequences underlined) = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

The PCR reaction was carried out using the following cycling conditions: an initial cycle at 95°C for 3 minutes followed by 45 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds and a final extension cycle at 72 °C for 5 minutes. The incorporation of dual indexes to the library in the subsequent 8 cycles of Index PCR step was carried out using primers from the Nextera XT Index Kit (Illumina Inc., San Diego. CA, USA) and enabled the sequencing of 96 samples in each setup. The samples were DNA quantified using the Qubit dsDNA HS Assay Kit (Life Technologies) and normalized to 4nM. The samples were pooled and prepared for 2 x 300 cycles of paired-end sequencing on the

Illumina Miseq sequencing platform using reagents from the Miseq reagent kit v3 (Illumina).

Statistics and bioinformatics

The chosen bioinformatic pipeline was Quantitative Insights Into Microbial Ecology (QIIME - http://qiime.org) version 1.9.1, run on Macintosh OSX using the MacQIIME package. Two files per sample from the Illumina MiSeq, one forward read, and one with reverse read was first joined with at least an overlap of 100 base pairs. The resulting files were merged to one library and sequences of poor quality were discarded, demaning a base quality score (phred score) of 19 or higher. Operational taxonomic units (OTUs) were picked using the open reference based approach in QIIME using Uclust with a 97% sequence similarity threshold (6) and GreenGenes version 13.8 as the reference database (7). All OTUs that constituted less than 0.0005% of the total sequence number were removed (8). The GreenGenes database (v.13.8) was also used for taxonomic assignment of OTUs (7) with the Ribosomal Database Project (RDP) classifier (9). All sequences from OTUs seen in corresponding negative control samples were deleted for the downstream analyses (10). Phylogenetic tree construction was performed with FastTree (11), after alignment using PyNAST (12).

In order to assess similiarity between samples obtained by different bronchoscopic sampling techniques and the oropharyngeal microbiome, the taxonomic distribution and diversity of OTUs from the OW samples were compared to all other sample types. Alpha-diversity was evaluated using Faith's phylogenetic diversity (PD), or “PD wholetree”. Beta-diversity was estimated with unweighted UniFrac, as well as visualization of

taxonomic distribution of OTUs and beta-diversity with principal coordinates analyses (PCoA) of UniFrac distance matrices for the entire data-set (13). Diversity analyses require a similar number of sequences in each sample, which was ensured by setting rarefaction levels. Samples with fewer sequences than the rarefaction level were excluded, whereas a number of sequences equal to the rarefaction level was chosen at random from the remaining samples. Due to the previous removal of a large number of sequences (the negative control sample OTUs), the rarefaction levels were relatively low. The proportion of taxa by sample type was tested using the *betafit* command in Stata as well as non-parametric trend tests. Statistical significance for alpha-diversity and beta-diversity between sampling methods was evaluated by Bonferroni-corrected Wilcoxon matched-pairs test in Stata version 13.2 (Statacorp, Texas, USA) and Bonferroni-corrected permutational ANOVA (permanova) with 1000 permutations in QIIME, respectively.

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FIGURE LEGENDS

Figure S1: Illustration of protected bronchoalveolar lavage (PBAL) with phosphate buffered saline (PBS).

Figure S2: Illustration of protected specimen brush (PSB) sampling.

Figure S3: Box-plot of alpha-diversity measured by wholetree phylogenetic differences by which lung that was sampled first and right/left protected specimen brush (PSB). Rarefied at 1000 sequences.

Figure S4: Principal coordinates analyses of unweighted UniFrac distances by which lung that was sampled first (red dots – left side first, blue dots – right side first) in right protected specimen brushes (PSB) from the right lower lobe and left PSBs from the left upper lobe. Rarefied at 1000 sequences.