Characterization of the Lower Airways and Oral Microbiota in Healthy Young Persons in the Community

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DNA extraction and PCR amplification

DNA was extracted from 500 µL sample aliquots using the DNeasy Blood & Tissue Kit (QIAGEN®, Toronto, ON, Canada). Each aliquot was centrifuged for 10 minutes at 5000 × g to form a pellet. After the supernatant was discarded, the pellet was resuspended in 180 µL of Buffer ATL and DNA was extracted according to the manufacturer's instructions· The concentration of DNA was measured using the Quant-iT® High-Sensitivity dsDNA Assay Kit (Life Technologies, Carlsbad, CA, USA).

Total 16S rRNA libraries were quantified using a droplet digital PCR assay (Bio-Rad Laboratories®, Mississauga, Canada). PCR amplification was performed using the AccuPrime® Taq DNA Polymerase System (Life Technologies®, Carlsbad, CA, USA). Primers targeting the variable V4 region of the 16S rRNA gene were used.

Each 20 µL PCR reaction contained 20 pmol of each indexed primer, 2 µL of 10× PCR Buffer II, 0.15 µL of Taq DNA Polymerase, and 20 ng of DNA template. PCR reactions were placed in a T100 Thermal Cycler (Bio-Rad Laboratories®, Mississauga, ON, Canada). The touchdown PCR program was as follows: 95°C for 2 min; 20 cycles of 95°C for 20 s, 60°C for 15 s (decreasing by 0.3°C per cycle), and 72°C for 90 s; 20 cycles of 95°C for 20 s, 55°C for 15 s, and 72°C for 90 s; and, lastly, 72°C for 5 min. The size of the obtained amplicons was checked by gel electrophoresis with a 1.5% agarose gel containing SYBR Safe DNA Gel Stain (Life Technologies®, Carlsbad, CA, USA). The presence of a band on the agarose gel indicated successful amplification. Before pooling the PCR amplicons, cleanup and normalization were performed using the SequalPrep® Normalization Plate Kit (Applied Biosystems®, Frederick, MD, USA). The pool of PCR reaction products was checked for size and quality using a DNA 1000 Kit with the 2100 Bioanalyzer system (Agilent Technologies®, Santa Clara, CA, USA) and diluted to 4 nM with 10 mM Tris-Cl pH 8.5 before sequencing.

Sequencing accuracy

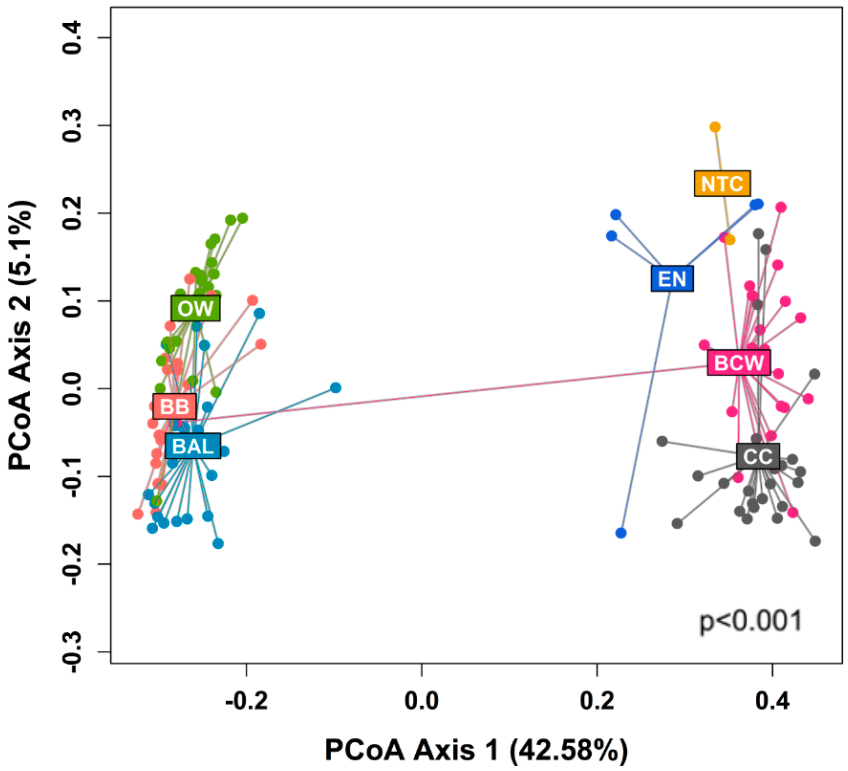
A mock community (HM-782D) containing 20 bacterial species was provided to evaluate the sequence quality (BEI Resources®, Virginia, USA). According to the *qiime quality-control evaluate-seqs* plugin function [1], we detected only 5 mismatches and no gaps (identified in only 2/20 ASVs from our MOCK community), resulting in a sequencing accuracy of ~99.5%.

Table S1: Taxonomic annotations at the phylum and genus levels for amplicon sequence variants (ASVs) identified as potential contaminants according to the Decontam R package.

ASV	Phylum	Genus	Species
ASV1	*Proteobacteria*	*Escherichia-Shigella*	NA
ASV2	*Cyanobacteria*	*Chloroplast*	NA
ASV3	*Cyanobacteria*	*Chloroplast*	NA
ASV4	*Proteobacteria*	*Sphingomonas*	NA
ASV5	*Proteobacteria*	NA	NA
ASV6	*Proteobacteria*	*Tepidiphilus*	*uncultured_bacterium*
ASV7	*Firmicutes*	*Mogibacterium*	*uncultured_rumen*
ASV8	*Actinobacteriota*	*Corynebacterium*	NA
ASV9	*Verrucomicrobiota*	*Chthoniobacter*	NA
ASV10	*Patescibacteria*	*Candidatus Lloydbacteria*	*Candidatus Lloydbacteria*
ASV11	*Firmicutes*	*Mogibacterium*	*uncultured rumen*
ASV12	*Cyanobacteria*	*Chloroplast*	NA
ASV13	*Proteobacteria*	*Brevundimonas*	NA
ASV14	*Proteobacteria*	*Bosea*	NA
ASV15	*Proteobacteria*	*Legionella*	NA
ASV16	*Proteobacteria*	*Neisseria*	NA
ASV17	*Verrucomicrobiota*	*Candidatus Xiphinematobacter*	*uncultured Candidatus*
ASV18	*Bdellovibrionota*	*Oligoflexus*	*metagenome*
ASV19	*Proteobacteria*	*Methylobacterium-Methylorubrum*	NA
ASV20	*Proteobacteria*	*Pedomicrobium*	
ASV21	*Patescibacteria*	*Candidatus Nomurabacteria*	*uncultured bacterium*
ASV22	*Patescibacteria*	*Candidatus Azambacteria*	*Parcubacteria bacterium*
ASV23	*Bacteroidota*	*Porphyromonas*	*uncultured bacterium*
ASV24	*Campylobacterota*	*Campylobacter*	NA
ASV25	*Firmicutes*	*Butyrivibrio*	*Eubacterium* sp.
ASV26	*Bacteroidota*	*Prevotella-7*	*Prevotella enoeca*
ASV27	*Cyanobacteria*	*Chloroplast*	NA
ASV28	*Firmicutes*	*Parvimonas*	*uncultured bacterium*
ASV29	*Bacteroidota*	*Tannerella*	*uncultured bacterium*
ASV30	*Desulfobacterota*	*Desulfobulbus*	NA

*Includes both control and clinical samples, as follows: 1) clinical samples: bronchial brushings, n=25; bronchoalveolar lavage (BAL) samples, n=25; oral wash samples, n=25; 2) control samples: extraction negatives, n=5; bronchoscope channel washes, n=23; CytoLyt controls, n=25. The "frequency" method in the Decontam R package [2] was used to identify possible contaminants; in this method, the frequency distribution of each sequencing feature as a function of the input DNA concentration is used to identify contaminants. Legend: NA = not applicable.

Figure S1: Comparison of microbial structures (beta-diversity) between clinical samples (BB, BAL, and OW) and control samples (EN, NTC, BCW, and CC).



Permutational Multivariate Analysis of Variance (PERMANOVA; function adonis in the vegan R package [3]) based on Generalized Unifrac distance (alpha=0.5) [4] was used for comparison of microbial structures among specimen types. Legend: PCoA = Principal Coordinates Analysis; BB = bronchial brushing (n=25); BAL = bronchoalveolar lavage (n=25); OW = oral wash (n=25); BCW = bronchoscope channel wash (n=23); EN = extraction negative (n=5); NTC = non-template control (n=2); CC = CytoLyt controls (n=25). The BCW controls were retrieved by flushing 40 mL of sterile 0,9% saline through the bronchoscope before the procedure into a sterile specimen cup. The EN controls contained only DNA extraction reagents, whereas NTC consisted of ultra-purified water instead of a DNA sample during the PCR reaction, and the CC controls contained only CytoLyt® solution. Pairwise PERMANOVA results are displayed in Table S2.

Table S2: Pairwise PERMANOVA results based on a Generalized Unifrac distance considering clinical samples (n=75) and control samples (n=55).

Group 1	Group 2	Sample size	Permutations	*p-value*	*adj. p-value*
BAL	BB	50	999	0.009	0.01
BAL	BCW	48	999	0.001	0.002
BAL	CC	50	999	0.001	0.002
BAL	EN	30	999	0.001	0.002
BAL	OW	50	999	0.001	0.002
BAL	NTC	27	999	0.003	0.005
BB	BCW	48	999	0.001	0.002
BB	CC	50	999	0.001	0.002
BB	EN	30	999	0.001	0.002
BB	OW	50	999	0.001	0.002
BB	NTC	27	999	0.002	0.003
BCW	CC	48	999	0.001	0.002
BCW	EN	28	999	0.013	0.013
BCW	OW	48	999	0.001	0.002
BCW	NTC	25	999	0.006	0.007
CC	EN	30	999	0.005	0.007
CC	OW	50	999	0.001	0.002
CC	NTC	27	999	0.008	0.009
EN	OW	30	999	0.001	0.002
EN	NTC	7	999	0.493	0.49
OW	NTC	27	999	0.004	0.006

Legend: BB = bronchial brushing (n=25); BAL = bronchoalveolar lavage (n=25); OW = oral wash (n=25); BCW = bronchoscope channel wash (n=23); EN = extraction negative (n=5); NTC = non-template control (n=2); CC = CytoLyt control (n=25). p-values were based on the Permutational analysis of variance (PERMANOVA) method [5], and pairwise PERMANOVA results were obtained according to the Benjamini-Hochberg method [6]. The PERMANOVA results were obtained directly from QIIME 2™ (based on the qiime diversity plugin) .

Table S3: Most abundant bacterial species identified in each microbiome compartment.

	Bronchial Brushings			BAL samples			Oral Wash samples		
Species	**Mean**	**Median**	**Q1; Q3**	**Mean**	**Median**	**Q1; Q3**	**Mean**	**Median**	**Q1; Q3**
*Veillonela dispar**	14.0	14.7	9.2; 7.9	16.1	15.5	13.9; 18.1	15.5	15.0	11.4; 16.8
*Haemophilus parainfluenzae**	4.2	3.1	1.7; 6.2	6.3	5.5	4.5; 7.2	15.3	16.3	11.7; 18.7
*Prevotella jejuni**	12.0	9.6	5.9; 17.5	9.2	8.8	6.9; 11.9	6.9	5.4	3.8; 18.7
Prevotella melaninogenica	7.4	7.0	2.6; 10.9	7.8	7.0	4.1; 9.1	11.1	10.9	6.7; 12.5
Prevotella pallens	8.4	6.1	3.1; 9.7	6.7	5.8	3.9; 9.3	5.4	4.0	3.1; 7.9
Megasphaera micronuciformis	3.0	2.7	0.3; 4.2	2.3	2.4	1.5; 3.2	0.6	0.5	0.2; 0.8
Prevotella salivae	2.5	2.1	1.0; 3.4	1.4	1.1	0.8; 2.1	1.5	1.1	0.5; 2.2
Streptococcus salivarius	0.5	0.3	0.1; 0.7	0.4	0.3	0.1; 0.5	0.3	0.1	0.1; 0.4
Mogibacterium pumilum	0.2	0.1	0.0; 0.2	0.3	0.2	0.1; 0.5	0.0	0.0	0.0; 0.0
Prevotella intermedia	0.9	0.1	0.0; 0.6	1.8	0.2	0.0; 1.1	0.8	0.1	0.0; 0.5
Prevotella nigrescens	1.2	0.1	0.0; 1.2	0.8	0.4	0.1; 0.9	0.8	0.3	0.2; 0.9

*Species identification only possible after performing sequence comparisons using the National Center for Biotechnology Information Basic Local Alignment Tool (NCBI BLAST, Maryland, USA) .

Definition of abbreviations: bronchoalveolar lavage; Q1: first quartile; Q3: third quartile.

Table S4: Relative abundance and frequency data of pathogenic genera in each microbiome compartment based on oral and lower airways samples collected from healthy subjects (n=25).

Taxon	MICROBIOME COMPARTMENT						Statistics
	Bronchial brushings		BAL samples		Oral wash samples		
Genus	**Median**	**Observed in**	**Median**	**Observed in**	**Median**	**Observed in**	***p-value****
Moraxella	0.0%	2/25 (8.0%)	0.0%	1/25 (4.0%)	0.0%	1/25 (4.0%)	<0.001
Pseudomonas	0.0%	4/25 (16.0%)	0.0%	2/25 (8.0%)	0.0%	0/25 (0.0%)	<0.001
Acinetobacter	0.0%	5/25 (20.0%)	0.0%	0/25 (0.0%)	0.0%	0/25 (0.0%)	<0.001
Staphylococcus	0.0%	5/25 (20.0%)	0.0%	0/25 (0.0%)	0.0%	0/25 (0.0%)	<0.001

Definition of abbreviations: BAL = bronchoalveolar lavage.
*P-values were calculated using a Fisher's exact test.

Table S5: Baseline characteristics of participants recruited into the British Columbia Cancer Agency (BCCA) cohort (n=47) [7].

Variables	Results
Age, years	62.9 ± 7.8
Sex, female	22 (46.8)
Smoking status	
Current smokers	20 (42.5)
Former smokers	24 (51.1)
Never-smokers	3 (6.4)*
Pack-years	47.0 ± 13.9
COPD status	
COPD	24 (51.1)
Non-COPD	23 (48.9)

Values are expressed as mean ± SD or n (%). Definition of abbreviations: COPD = Chronic Obstructive Pulmonary Disease.
*Out of the three never-smokers, two were non-COPD.

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