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Early View

Research letter

The airway microbiota in children newly diagnosed with bronchiectasis largely retains its diversity

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The airway microbiota in children newly diagnosed with bronchiectasis largely retains its diversity

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To the Editor

There is a great deal of interest in the airway microbiota, its diversity and the role of specific microbial taxa in the pathophysiology of lung disease [1]. Non-cystic fibrosis bronchiectasis is a significant public health problem in many countries including New Zealand where prevalence is high and morbidity and mortality are substantial [2-4]. A role for bacteria in the pathophysiology of bronchiectasis is widely accepted but poorly characterised due to inherent difficulties with lower airway sampling, especially in young children. Culture-based studies demonstrate associations with *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae* in children [2,5] and *Pseudomonas aeruginosa* in adults and those with severe disease [6,7]. Culture-independent methods have revealed complex airway microbial communities with differences between children and adults with bronchiectasis, and between adults with cystic fibrosis (CF) and bronchiectasis [8]. A shared core microbiota was reported for children with bronchiectasis, protracted bacterial bronchitis (PBB) and CF, with most of these taxa also seen in healthy controls [8]. Subsequent research indicated that microbiota composition could be used to distinguish bronchiectasis, PBB and control children [9]. In children with PBB, bronchial brush samples were dominated by *Haemophilus, Moraxella, Streptococcus* and *Neisseria* [10], while bronchoalveolar lavage (BAL) samples showed that *Bacteroides* and *Haemophilus* were more common than in disease controls, with *Lactococcus* and *Lactobacillus* less common [11]. Differences in study findings could be due to methodological differences, disease heterogeneity and timing of airway samples. Adults with bronchiectasis demonstrate a dominance of either *Haemophilus* or *Pseudomonas*. Pseudomonal dominance is associated with end-stage lung disease and potentially adverse clinical outcomes [6,7]. Recent longitudinal studies in CF children demonstrate a reduction in

microbial diversity with age, with establishment of more traditional disease-modifying taxa [12]. An emerging common theme is that reduced microbiota diversity, and certain bacterial taxa, may potentially be associated with adverse clinical outcomes.

We undertook a prospective study at Starship Children's Hospital, Auckland, New Zealand between 2015-2017, enrolling children being investigated for bronchiectasis as per our regional guidelines [13]. Flexible bronchoscopic BAL (1 mL/kg/lavage of 0.9% saline, maximum 20 mL) was performed from the 2-3 most affected lobes in children with bronchiectasis as identified by chest comptuted tomography (CT) scan. Bronchiectasis was radiologically defined as a broncho-arterial ratio of more than 1.0 in combination with other well-described radiological features [2,14]. We also recruited controls undergoing elective surgery, with no respiratory history. A single non-bronchoscopic lavage was performed in the controls. To assess potential contamination introduced from the bronchoscope, triplicate bronchoscopy negative controls were conducted. DNA was extracted using the Qiagen AllPrep DNA/RNA Mini Kit, and PCR-amplified bacterial 16S rRNA genes (V3-V4 region) sequenced using Illumina MiSeq [15]. Bacterial load was estimated using real-time PCR quantification of 16S rRNA gene copies. Negative (no-template) PCR controls and extraction kit controls using sterile water were performed.

Patient data were compared using a Chi-squared test for categorical variables and Kruskal-Wallis test for continuous variables. Sequence and statistical analyses utilised USEARCH, QIIME and R to calculate diversity metrics based on 97% 16S rRNA gene-defined bacterial operational taxonomic units (OTUs) [15]. Sequence data were deposited in the NCBI Sequence Read Archive (PRJNA 422718).

Thirty-two children (21 male and 11 female) with bronchiectasis (median age 2.3 years, range 0.9-16 years) and 11 (7 male and 4 female) healthy controls (median 5.9 years, range 1.4-13.1 years) were recruited. There were significant differences (p<0.05) between groups, with the bronchiectasis cohort having an over-representation of Maori (21, 65.6%) and Pasifika (6, 18.8%) children, more tobacco smoke exposure and coming from areas of higher social deprivation, compared to controls. The aetiology of bronchiectasis after extensive work-up was post-infectious (15, 46.9%), idiopathic (10, 31.3%) and aspiration related (5, 15.6%). Bilateral bronchiectasis was seen in 26 (81.3%) children, with three or more affected lobes in 21 (65.6%) children.

We obtained 2,214,195 high-quality 16S rRNA gene sequences in total, with 83 bacterial genera identified across 188 OTUs. Overall, *Proteobacteria* (mean relative sequence abundance ±S.D.: 62.6±28.8%), *Firmicutes* (21.1±22.5%), and *Bacteroidetes* (9.4±8.6%) were the dominant bacterial phyla in children with bronchiectasis. The most abundant genera, which were frequently represented by multiple OTUs, were *Haemophilus* (36.7±35.9%), *Streptococcus* (16.7±21.1%), *Neisseria* (14.7±14.2%), *Moraxella* (9.32±17.8%) and *Fusobacterium* (3.85 ± 9.6%) (Figure 1A). OTUs representing *Haemophilus, Neisseria* and *Streptococcus* were also prominent in healthy controls. Inter-individual differences in children with bronchiectasis were considerable, explaining 49% of observed microbiota variation (PERMANOVA, R^2=0.49, p=0.001), while intra-individual differences were minimal (Figure 1A). Bacterial community richness and diversity did not differ significantly (p>0.05) between children with bronchiectasis and controls across multiple indices, namely Chao1, Shannon, Simpson's index, number of observed OTUs, and Simpson's evenness measure E (Figure 1B). Non-metric multidimensional scaling

plots reaffirmed the high degree of overlap in microbiotas of controls and children with bronchiectasis (Figure 1C), while Dunn's test with Bonferroni and false discovery rate corrections revealed no significant differences in OTU relative sequence abundance between the cohorts. Real-time PCR indicated no significant difference in lower airway bacterial load between groups (Wilcoxon rank sum test $p>0.05$).

Consistent with the small number of studies in children, *Haemophilus*, *Streptococcus*, *Moraxella* and *Neisseria* were the most abundant taxa in lower airways of our children with bronchiectasis. Measures of alpha and beta diversity were also similar between children with bronchiectasis and healthy controls. This is in contrast with adults with bronchiectasis who additionally harbour *Pseudomonas* and *Staphylococcus* [7]. A study of bacterial communities in resected lung tissue demonstrated that, in advanced disease, the microbiota of bronchiectasis was similar to that CF, with Proteobacteria being the most dominant phylum and *Pseudomonas* being the most prevalent genus [6]. Thus, current evidence suggests that airway microbiota in some airway diseases and especially bronchiectasis is dynamic and evolves over time. This, and the association of microbiota and disease outcomes need further evaluation.

One limitation of our study is the relatively small number of children involved, although our numbers are comparable to other recent studies and reflect challenges in obtaining paediatric lower airway samples. The strengths of this study include prospective recruitment of children, targeted lower airway sampling in children with CT-diagnosed bronchiectasis and healthy controls, and successful characterisation of the lower airway microbiota in a condition for which few data are available. While one recent study showed that a combination of nasopharyngeal and oropharyngeal

sampling provided a representation of the lower airway microbiota, upper airway samples nonetheless still differed significantly from BAL samples [9]. Thus, in our opinion, lavage remains the gold standard for accurately studying lower airway disease. Importantly, bronchoscopy controls, extraction and no-template PCR controls yielded a negligible number of 16S rRNA gene sequences, giving us confidence in the integrity of our obtained data.

Our data suggest that, at the point of diagnosis of bronchiectasis, lower airway bacterial diversity is largely preserved, similar to that of healthy controls, and changes associated with progressive disease are not yet present. If this diversity can continue to be maintained, we speculate that this could limit disease progression. Future studies, preferably incorporating a longitudinal aspect, should focus on disease progression in relation to microbiota changes, as well as the function of the microorganisms in question.

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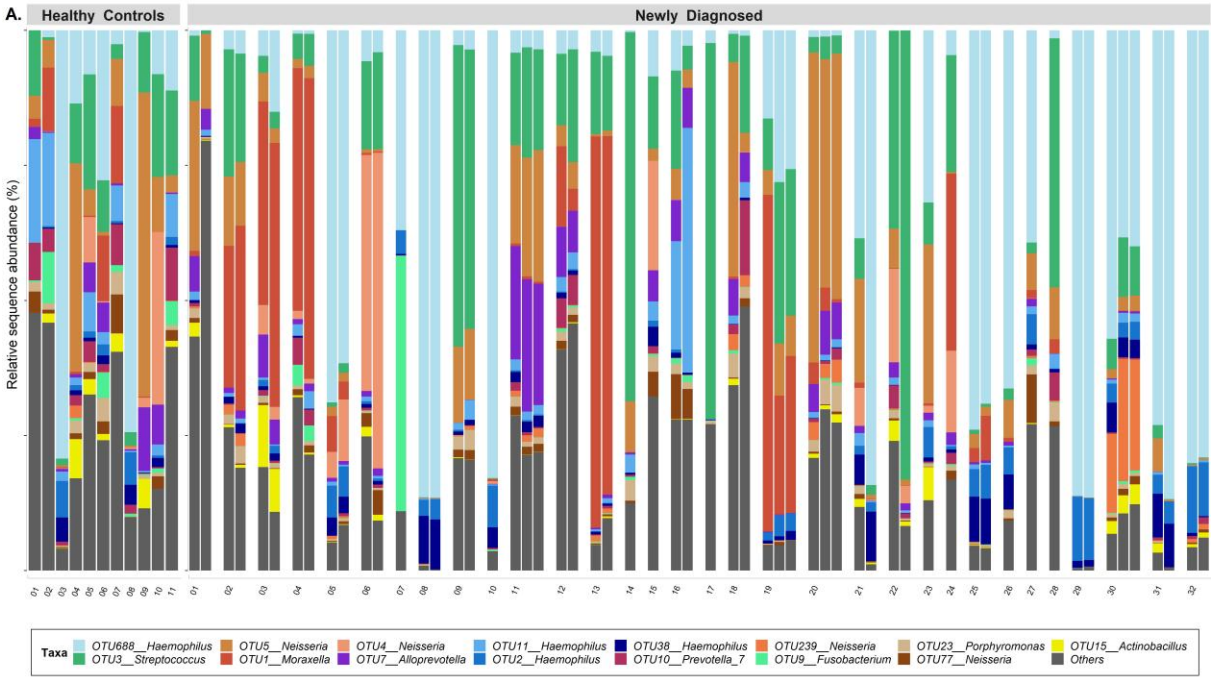
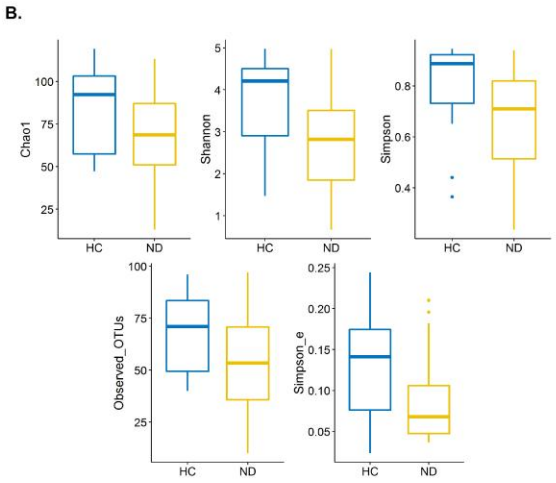
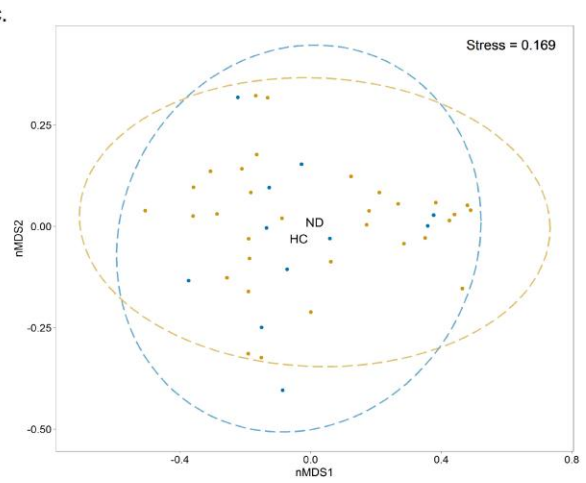
Figure legend:

Figure 1. Bacterial community composition and diversity for newly diagnosed bronchiectasis (ND, n=32) and healthy controls (HC, n=11). (A) Relative abundances of taxon-assigned OTUs at 97% 16S rRNA gene sequence similarity in lavage samples taken from healthy controls and newly diagnosed bronchiectasis. The 15 most abundant OTUs are shown, with all remaining taxon-assigned OTUs grouped in "Others". (B) Alpha diversity comparisons between cohorts. Median values are indicated by the solid line within each box, and the box extends to upper and lower quartile values; outliers are indicated by closed circles. (C) Beta diversity visualised using non-metric multidimensional scaling (nMDS) of the Bray-Curtis dissimilarity metric. Ellipses represent the mean of the description coordinates at the center, with dispersion of the ellipses calculated using the standard deviation of the weighted average of covariance matrix group scores.


A.
Healthy Controls
Newly Diagnosed
Relative sequence abundance (%)
Taxa
OTU688__Haemophilus
OTU3__Streptococcus
OTU5__Neisseria
OTU1__Moraxella
OTU4__Neisseria
OTU7__Alloprevotella
OTU11__Haemophilus
OTU2__Haemophilus
OTU38__Haemophilus
OTU10__Prevotella_7
OTU239__Neisseria
OTU9__Fusobacterium
OTU23__Porphyromonas
OTU77__Neisseria
OTU15__Actinobacillus
Others


B.
Chao1
Shannon
Simpson
Observed_OTUs
Simpson_e
HC
ND


C.
Stress = 0.169
nMDS1
nMDS2
ND
HC