Directly Sampling the Lung of a Young Child with Cystic Fibrosis Reveals Diverse Microbiota

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Abstract

Rationale: The airways of people with cystic fibrosis (CF) are chronically infected with a variety of bacterial species. Although routine culture methods are usually used to diagnose these infections, culture-independent, DNA-based methods have identified many bacterial species in CF respiratory secretions that are not routinely cultured. Many prior culture-independent studies focused either on microbiota in explanted CF lungs, reflecting end-stage disease, or those in oropharyngeal swabs, which likely sample areas in addition to the lower airways. Therefore, it was unknown whether the lower airways of children with CF, well before end-stage but with symptomatic lung disease, truly contained diverse microbiota.

Objectives: To define the microbiota in the diseased lung tissue of a child who underwent lobectomy for severe, localized CF lung disease.

Methods: After pathologic examination verified that this child's lung tissue reflected CF lung disease, we used bacterial ribosomal RNA gene pyrosequencing and computational

phylogenetic analysis to identify the microbiota in serial sections of the tissue.

Measurements and Main Results: This analysis identified diverse, and anatomically heterogeneous, bacterial populations in the lung tissue that contained both culturable and nonculturable species, including abundant *Haemophilus*, *Ralstonia*, and *Propionibacterium* species. Routine clinical cultures identified only *Staphylococcus aureus*, which represented only a small fraction of the microbiota found by sequencing. Microbiota analysis of an intraoperative oropharyngeal swab identified predominantly *Streptococcus* species. The oropharyngeal findings therefore represented the lung tissue microbiota poorly, in agreement with findings from earlier studies of oropharyngeal swabs in end-stage disease.

Conclusions: These results support the concept that diverse and spatially heterogeneous microbiota, not necessarily dominated by "traditional CF pathogens," are present in the airways of young, symptomatic children with early CF lung disease.

Keywords: cystic fibrosis; microbiota; lobectomy; infection

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Cystic fibrosis (CF) lung disease is characterized by chronic infection with diverse microbes, a handful of which are traditionally associated with pathogenesis (1). These "standard CF

pathogens" are culturable by routine methods; examples include *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Burkholderia*

cepacia complex, and *Achromobacter* species. Therefore, standard antibiotic therapy for CF lung infections is typically chosen to target these organisms (2).

A growing number of studies using culture-independent, DNA-based methods have identified greater microbial diversity in CF respiratory secretions than is identified by culture. For example, culture-independent studies of oropharyngeal swabs, sputum, and bronchoalveolar lavage (BAL) fluid identified microbes that are either rarely detected or difficult to identify by routine culture techniques (3–6), including anaerobic species, often at abundances similar to or exceeding those of traditional pathogens. These findings raise important questions regarding the microbial determinants of CF lung disease, and the broader effects of antibiotics.

Many of the bacterial species newly identified in respiratory specimens may also be inhabitants of the oropharynx or gastrointestinal tract. As the previously mentioned specimen types likely sample, and therefore reflect, both the lungs and these nonlung sources to varying degrees (7), CF microbiota studies have examined lungs collected during transplantation or postmortem (therefore representing end-stage disease [7–10]), and have yielded some consistent and some divergent findings. For example, two studies identified low-diversity microbiota in explanted lungs, each dominated by one or two traditional pathogens, but relatively higher diversity microbiota in concurrent oropharyngeal and sputum samples, with sputum identifying the predominant organisms in the lung more accurately than did oropharyngeal samples (7–9). By contrast, other studies of explanted and postmortem lungs demonstrated more diverse microbiota, including anaerobes (except where specified otherwise, the word “diverse” here signifies the number of species, often referred to as “richness” in ecology) (10, 11). However, because these specimens reflected end-stage CF lung disease, it is difficult to interpret the relevance of their lung microbiota findings for earlier disease. Prior CF microbiota studies using oropharyngeal samples (5) and sputum (12) before end-stage suggested gradual decreases in diversity in those specimens with increasing patient age and advancing disease. Therefore, whether the relatively low-diversity lung tissue communities reflected the end-stage result of this “simplification,” whether oropharyngeal samples collected from children before end-stage primarily

reflected microbiota from nonlung sources without accurately depicting lower airway diversity, or whether a more complex model accounted for these results, could not be determined.

We applied culture-independent molecular methods to define the microbiota in lung tissue from a very young child with CF who underwent lobectomy. As lobectomies are uncommon in children with CF, this event presented a rare opportunity to directly study CF lung microbiota earlier than is usually possible. The resulting findings provide a valuable link between lung tissue and respiratory secretion microbiota studies, indicating that diseased CF lung microbiota can be both diverse and represented poorly by cultures or by sequencing of concurrent oropharyngeal specimens.

Methods

Human Subjects

Specimens were collected with approval by the Seattle Children’s Hospital (Seattle, WA) Institutional Review Board. Parental consent for specimen collection and use for research was obtained before the surgical procedure.

Specimen Culture

Standard aerobic and anaerobic cultures were performed on ground lung tissue. Quantitative, CF-specific cultures of secretions from lung tissue and tracheal aspirates used secretions (0.5 g) vortexed with 0.5 ml of Sputolysin (Calbiochem, La Jolla, CA). Oropharyngeal swabs were vortexed with 0.5 ml of sterile saline and 0.5 ml of Sputolysin. Serial dilutions in phosphate-buffered saline were plated onto selective media recommended for CF cultures: MacConkey, oxidation-fermentation polymyxin-bacitracin-lactose, DNase, and mannitol salt agars, which were incubated at 35°C in air for 48 hours; Selective Strep and *Haemophilus* selective agars, which were incubated anaerobically at 35°C for 48 hours; and *Candida* BCG agar, which was incubated at 30°C in air for at least 3 days. The number of colony-forming units per gram (cfu/g) was determined by colony counts on each plate for sputum. Identification of organisms was by standard microbiology techniques, including biochemical testing, polymerase chain reaction (PCR), and sequencing as appropriate, as described (13).

Lung Section DNA Extraction

Lung sections were received on ice and stored at –80°C until required. The samples were then thawed on ice and DNA was extracted from the sections, using a PowerSoil DNA isolation kit (MO BIO, Carlsbad, CA) with modifications to optimize DNA yield. Briefly, each lung section was added to an individual 2-ml tube containing 1.0 g of sterile, DNA-free zirconia/silica beads and 750 µl of PowerLyzer PowerSoil bead solution and 60 µl of solution C1 (MO BIO). The tube was incubated at 65°C for 10 minutes, and then at 95°C for another 10 minutes. The mixture was vortexed for 10 minutes. All the remaining steps were performed as described by the manufacturer. The DNA sample was stored at –80°C until required.

Oropharyngeal Swab DNA Extraction

Bacterial DNA from a cotton oropharyngeal swab was extracted with the MO BIO soil extraction kit, using lysis buffer and ceramic beads according to the kit instructions.

No-Template (Buffer-Only) Control Extraction

The no-template control was produced with the same kit and bead manufacture lot as used for the previous DNA extractions. Briefly, an individual 2-ml tube containing 1.0 g of sterile, DNA-free zirconia/silica beads and 750 µl of PowerLyzer PowerSoil bead solution and 60 µl of solution C1 (MO BIO) was incubated at 65°C for 10 minutes and then at 95°C for another 10 minutes. The mixture was vortexed for 10 minutes. All the remaining steps were performed as described by the manufacturer. The sample was stored at –80°C until sequencing.

Processing of Lobectomy Tissue for Pathology

The right lower lobe was received and processed within 1 hour of resection. On receipt it was transversely sectioned from the hilum to the periphery. Secretions and tissue from the most proximal section were cultured as described previously. Lung tissue containing bronchi from various regions was flash frozen in liquid nitrogen and stored at –80°C until analysis. Sections directly adjacent to frozen lung were fixed in 10% formalin and paraffin-embedded for routine sectioning and hematoxylin-eosin staining before pathologic review.

Bacterial Tag-Encoded FLX Amplicon Pyrosequencing

The bacterial microbiota of each sample were defined by bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) as described previously (14, 15). For lung section 1, 10,000-read coverage was requested (see Table E1 in the online supplement); microbiota analysis demonstrated that 3000 reads would have provided sufficient coverage, and 3000 reads on average were requested for subsequent sections. In brief, primers 28F (5'-GAGTTTGATCCTGGCTCAG) and 519r (5'-GTNTTACNGCGGCKGCTG) were used to amplify an approximate 500-bp fragment of the 16S ribosomal RNA (rRNA) gene covering the V1–V3 variable regions. Analysis of V1–V3 has been shown to provide putative taxonomic resolution to the species level (7, 16). Generation of the sequencing library involved a one-step PCR of 30 cycles, using a mixture of Hot Start and HotStar high-fidelity *Taq* polymerase (Qiagen, Valencia, CA). bTEFAP analyses used a Roche 454 FLX instrument with Titanium reagents, performed at the MR DNA Laboratory (Shallowater, TX) according to established protocols (www.mrdnalab.com). Bacterial sequences were assigned taxonomic identities, and the resulting data were analyzed via subsampling for diversity measures, using the mothur (17) (version 1.33.3) software package for sequence processing as follows.

Sequence Analytical Pipeline

Sequence data were derived by sequencing the V1–V3 region of the 16S rRNA gene as described (7, 14, 15) at MR DNA Laboratory (www.mrdnalab.com). Sequences were trimmed of barcodes and primers; sequences less than 200 bp in length were removed. Sequences with ambiguous base calls and sequences with homopolymer runs exceeding 8 bp were also removed from the data set. Sequences were then denoised, and both chimeric sequences and misamplified eukaryotic sequences (18) were removed. Good's coverage was calculated to estimate the adequacy of the coverage provided by the number of reads considered as described (19) (Table E1). The remaining high-quality bacterial 16S rRNA gene sequence reads (Table E1) were processed for bacterial phylogeny. Species-level operational taxonomic units (OTUs) were defined,

clustering at 3% divergence (97% similarity) (14, 15, 20–24). OTUs were then taxonomically classified, using BLASTn against a curated GreenGenes 16S rRNA gene sequence database (<http://greengenes.lbl.gov>) (25) and compiled by taxonomic level into both “counts” and “percentage” files. Sequences with more than 97% identity to annotated rRNA gene sequences were considered to be resolved to the species level; those with identities between 95 and 97%, to the genus level; those between 90 and 95%, to the family level; those between 85 and 90%, to the order level; those between 80 and 85%, to the class level; and those between 77 and 80% were reported to the phylum level (Tables E1–E3). Resulting taxonomic data are presented to reflect all bacteria that represented more than 1% of bacterial reads in any sample in Table E2 and those that were identified by any reads in Table E3.

Diversity Analysis

To control for differences in sequencing depth between different sections of lung tissue, the reads for all sections were subsampled to the lowest number of reads yielded by sequencing for any of the sections (section 5, Table E1) before calculating diversity measures. Subsampling did not appreciably change the microbiota in any section (data not shown). α diversity (measures of microbiota diversity within each lung tissue section) was calculated using the Shannon index of diversity (H'), evenness was calculated on the basis of the Shannon index calculations, and richness was based on taxonomic identities in each sample (Table E4) (26). β diversity (measures of microbiota differences and similarities among the lung tissue sections) was calculated with the PRIMER software package (version 6) (27). This analysis included hierarchical group average clustering of a Bray–Curtis similarity matrix of 4th root-transformed data (Figure E1). The significance of subgroup structures was determined using a similarity profile (SIMPROF) test as described (28).

Results

Case Report

A 3-year-old child diagnosed with CF in infancy (homozygous F508del CFTR genotype) presented for surgical lobectomy

because of progressive, localized bronchiectasis and chronic lobar collapse.

The patient had an unremarkable birth and early infancy. By 3 months, the patient had developed persistent, severe cough and episodes of respiratory distress that responded variably to multiple courses of antibiotics targeting *S. aureus* and *H. influenzae*, which were each cultured from both oropharyngeal and BAL samples. *P. aeruginosa* was cultured intermittently from early oropharyngeal swabs but was not detected in any culture (i.e., three oropharyngeal and one BAL specimen) over the year before surgery.

Chest X-rays demonstrated occasional, right-sided opacification during the first 2 years of life. By 23 months of age, examinations revealed persistent right middle lobe opacification (Figure 1a); right-sided rales; and nighttime hypoxemia that did not improve with frequent chest physiotherapy, dornase, and both intravenous and oral antibiotics. Chest computed tomography (Figure 1b) identified dense atelectasis and bronchiectasis limited to the right middle and lower lobes (RML, RLL).

Because of the severity of disease, the patient was referred for removal of the severely bronchiectatic lobes and treated with intravenous ampicillin/sulbactam for 17 days before the procedure. On thoracotomy, the RLL was intensely inflamed, scarred, and friable, bleeding profusely during the procedure. Because of the resulting clinical instability, only the RLL was removed. The patient was discharged from the hospital 15 days after surgery, judged to be in stable and improved clinical condition.

Pathologic and Microbiological Analysis

Immediately after surgery, the resected RLL was preserved and sectioned (Figure 2a). Pathologic examination demonstrated grossly bronchiectatic airways with necrotizing airway-centric inflammation throughout (Figure 3). The bronchioles were uniformly obstructed by copious, inspissated mucus admixed with neutrophils and granular debris. The examination was consistent with severe, focal CF lung disease.

Four clinical cultures were performed on the day of surgery, including an oropharyngeal swab identifying 1+ *Serratia marcescens* and 1+ *Candida lusitanae*;

Figure 1. Radiographs of the patient at 33 months, 2 months before right lower pulmonary lobectomy. (a) Chest X-rays (posteroanterior and lateral), revealing dense right lower lobe and some right middle lobe atelectasis (arrow), with mediastinal shift to the right and hyperexpansion of the left upper lobe. (b) Chest computed tomography, illustrating dense right lower lobe atelectasis with extensive bronchiectasis.

a tracheal aspirate identifying 720 cfu/g methicillin-susceptible *S. aureus* (MSSA), 20 cfu/g *S. marcescens*, and *C. lusitaniae*; bronchial secretions from the most proximal lung section (section 1; Figure 2a), which contained airways visibly obstructed with secretions continuing into all distal sections, identifying only 40 cfu/g MSSA; and ground lung tissue from that same lung section, identifying MSSA and *C. lusitaniae*.

The former three cultures were processed aerobically primarily for standard CF pathogens; the latter specimen was cultured aerobically and anaerobically.

In contrast to cultures, bacterial 16S ribosomal RNA gene sequencing of lung section 1 identified many bacterial species (Figure 2b and Tables E1–E3), dominated by *H. influenzae* (76.2%), *Propionibacterium acnes* (8.5%), and

Staphylococcus epidermidis (4.1%), none of which were identified by culture; *S. aureus* comprised only 2.0% of sequence reads. Control, buffer-only samples run through the same extraction and analysis yielded only four sequence reads.

Analysis of the microbiota within five serially distal lung sections (Figures 2a and 2b) identified populations different from those in section 1; this heterogeneity is consistent with some previous adult lung explant findings (10). The predominant bacterial species identified were *Ralstonia pickettii* and *P. acnes* (Figure 2b and Tables E2 and E3). These two organisms, both identified as common in prior CF microbiota studies (3, 29–31), were not detected in any of the cultures. Taxa of the genus *Anaerococcus*, which, like *Propionibacterium*, includes anaerobes commonly found in CF sputum (31), were also detected in each section. Although the reason for the differences we observed between the microbiota from the proximal versus the more distal sections (Figure 2, and Tables E2 and E3) cannot be determined from our data, the proximal region of the plug may have differed from the distal portions either physicochemically or in its exposure to bacteria being delivered by inhalation. Despite yielding fewer bacterial reads than section 1, the data from sections 2–6 provided sufficient coverage to identify the dominant species therein (Table E1), as indicated both by calculations of Good's coverage and by the relative similarity in the nondominant bacteria detected in those sections (Figure 2).

The microbiota detected by sequencing in the intraoperative oropharyngeal swab were dominated by *Streptococcus* species (51.1%) and included none of the most abundant organisms identified in lung tissue and secretions by either culture or sequencing.

Diversity Analysis

Microbial diversity within (α diversity) and between (β diversity) lung tissue specimens was defined using several measures, including richness, or the number of taxa detected; evenness, a measure of relative abundance (i.e., whether one or a few species dominate); and Shannon index, which accounts for both the number and distribution of taxa. These analyses were performed after computationally subsampling the sequence data sets from

Figure 2. (a) Anatomy and (b) microbiota of the lung tissue removed from a young child with cystic fibrosis. (a) Schematic of the right lower lobectomy sample, indicating the locations of tissue sections that were analyzed by sequence-based microbiota methods. Section 1 was analyzed by both postoperative culture and sequencing; the remaining sections were analyzed by sequencing only. (b) Bar graph indicating the relative abundances and identities of the species detected by bacterial 16S ribosomal RNA gene sequencing in each lung tissue section and the intraoperative throat swab. Bacterial species shown in the legend comprised at least 1% of bacterial reads detected within an individual sample.

each tissue section to yield the same number of sequence reads for each section (585, the minimum read number per section; Table E1). As shown in Table E4, richness ranged from 11 to 25 species per lung tissue section, and the Shannon index ranged from 0.643 to 1.581. Shannon indices from this analysis were unrelated to sample weight (Tables E1 and E4), confirming that normalization by subsampling minimized bias due to size variation among tissue sections. Investigation of the overall similarity

among tissue section microbiota, measured by hierarchical group average cluster analysis (HCAN) and the similarity profile (SIMPROF) test, showed that the microbiota in section 1 were significantly dissimilar to those in sections 2–6 (SIMPROF $\pi = 3.86$; $P < 0.02$; Figure E1).

Discussion

These culture-independent results suggest that the lung tissue of a young child with

severe CF lung disease contained diverse and spatially heterogeneous bacterial populations dominated by species identified neither by cultures of lung tissue nor tracheal aspirates, nor in oropharyngeal specimens by any method. The lung microbiota identified differed from those found in earlier studies of end-stage lung tissue, which were often dominated by *P. aeruginosa* and *Achromobacter xylosoxidans* (7, 9); for comparison, the average diversity detected in this child's lung tissue was $H' = 1.14$, in the same range as those reported previously for CF sputum (12), but higher than values reported for microbiota in end-stage CF lung tissue ($H' < 0.25$) (7).

Our study has several limitations, including the unusual clinical presentation of our single study subject and the fact that we did not use sample-processing methods shown to increase detection of *Staphylococcus* species (26). Routine CF-specific culture methods did not detect bacterial taxa that were identified by sequencing; in the case of *Propionibacterium*, coagulase-negative *Staphylococcus*, and some *Streptococcus* species (all of which have been identified in CF secretions previously [3, 31]), this may be either because of the relative insensitivity of routine laboratory culture

Figure 3. Sections of the right lower lobe, illustrating bronchiectatic airways (arrows) filled with mucus and with necrotizing airway wall inflammation. Hematoxylin and eosin stain. Original magnification: left panel, $\times 40$; right panel, $\times 200$.

for these and other species, or because, for CF-specific cultures, clinical laboratories usually label these and other species as “oral flora” and do not report them when cultured. Alternatively, perioperative antibiotic exposure could have limited culture detection of these organisms. In addition, studies in CF (32) and in other conditions (33) have used molecular methods to identify in clinical specimens *H. influenzae* that was not identified by concurrent culture, as was the case here. In the case of *Ralstonia pickettii*, which has also been identified previously in CF secretions (30), this species was likely not cultured here as a result of its heterogeneous lung tissue distribution, and its rarity in the only section that was cultured (section 1; Table E3). The pathological processing of this specimen generated sections that differed in weight (Table E1) and therefore in the amount of infected tissue analyzed, potentially biasing the microbiota and diversity results. However, subsampling resulted

in little effect on microbiota composition or diversity, indicating that this was not a source of appreciable bias in this analysis.

Despite these limitations, our findings suggest that even diseased CF lungs are not necessarily infected with high densities of “traditional CF pathogens.” These findings support culture-based studies of children with CF (34–36) and the CF porcine and ferret models (37, 38), which identified respiratory symptoms, structural lung disease, and airway inflammation in the absence of abundant “traditional CF pathogens.” They also support prior lung explant studies, showing that oropharyngeal swabs did not accurately reflect the microbiota in concurrent lung samples. In one such study, sputum samples (which are not generally produced by young children) did identify the predominant lung organisms, but the microbiota detected in end-stage lungs were dominated by “traditional pathogens” usually detected

and reported by culture (7). Although the microbes detected from oropharyngeal swabs could serve as useful biomarkers of clinical outcomes, the disparities between results from culture and sequencing, and between oropharyngeal swabs and lung tissue, found in this and prior studies raise questions about how best to sample CF lower airway microbiology in early disease, particularly before expectoration. Future studies of additional CF lung explants from young patients, comparing the results with sputum for those patients who can expectorate or with BAL samples for those who cannot, could clarify the best sampling approach. The detection here of diverse, spatially heterogeneous microbiota in the diseased lung of a 3-year-old child with CF underscores the need for more research focusing on early CF lung infection and disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

- Sibley CD, Surette MG. The polymicrobial nature of airway infections in cystic fibrosis: Cangene Gold Medal Lecture. *Can J Microbiol* 2011; 57:69–77.
- Döring G, Flume P, Heijerman H, Elborn JS; Consensus Study Group. Treatment of lung infection in patients with cystic fibrosis: current and future strategies. *J Cyst Fibros* 2012;11:461–479.
- Harris JK, De Groot MA, Sagel SD, Zemanick ET, Kapsner R, Penvari C, Kaess H, Deterding RR, Accurso FJ, Pace NR. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. *Proc Natl Acad Sci USA* 2007;104:20529–20533.
- Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, Wolfgang MC. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. *PLoS One* 2012;7:e45001.
- Cox MJ, Allgaier M, Taylor B, Baek MS, Huang YJ, Daly RA, Karaoz U, Andersen GL, Brown R, Fujimura KE, et al. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. *PLoS One* 2010;5:e11044.
- Lynch SV, Bruce KD. The cystic fibrosis airway microbiome. *Cold Spring Harb Perspect Med* 2013;3:a009738.
- Goddard AF, Staudinger BJ, Dowd SE, Joshi-Datar A, Wolcott RD, Aitken ML, Fligner CL, Singh PK. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. *Proc Natl Acad Sci USA* 2012; 109:13769–13774.
- Rudkjøbing VB, Thomsen TR, Alhede M, Kragh KN, Nielsen PH, Johansen UR, Givskov M, Højby N, Bjarnsholt T. True microbiota involved in chronic lung infection of cystic fibrosis patients found by culturing and 16S rRNA gene analysis. *J Clin Microbiol* 2011;49: 4352–4355.
- Rudkjøbing VB, Thomsen TR, Alhede M, Kragh KN, Nielsen PH, Johansen UR, Givskov M, Højby N, Bjarnsholt T. The microorganisms in chronically infected end-stage and non-end-stage cystic fibrosis patients. *FEMS Immunol Med Microbiol* 2012; 65:236–244.
- Willner D, Haynes MR, Furlan M, Schmieder R, Lim YW, Rainey PB, Rohwer F, Conrad D. Spatial distribution of microbial communities in the cystic fibrosis lung. *ISME J* 2012;6:471–474.
- Blainey PC, Milla CE, Cornfield DN, Quake SR. Quantitative analysis of the human airway microbial ecology reveals a pervasive signature for cystic fibrosis. *Sci Transl Med* 2012;4:153ra130.
- Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, Cavalcoti JD, VanDevanter DR, Murray S, Li JZ, et al. Decade-long bacterial community dynamics in cystic fibrosis airways. *Proc Natl Acad Sci USA* 2012;109:5809–5814.
- Qin X, Emerson J, Stapp J, Stapp L, Abe P, Burns JL. Use of real-time PCR with multiple targets to identify *Pseudomonas aeruginosa* and other nonfermenting gram-negative bacilli from patients with cystic fibrosis. *J Clin Microbiol* 2003;41:4312–4317.
- Dowd SE, Sun Y, Wolcott RD, Domingo A, Carroll JA. Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned *Salmonella*-infected pigs. *Foodborne Pathog Dis* 2008;5:459–472.
- Dowd SE, Callaway TR, Wolcott RD, Sun Y, McKeenhan T, Hagevoort RG, Edrington TS. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiol* 2008;8:125.
- Kistler JO, Booth V, Bradshaw DJ, Wade WG. Bacterial community development in experimental gingivitis. *PLoS One* 2013;8: e71227.
- Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;75:7537–7541.
- Huys G, Vanhoutte T, Joossens M, Mahious AS, De Brandt E, Vermeire S, Swings J. Coamplification of eukaryotic DNA with 16S rRNA gene-based PCR primers: possible consequences for population fingerprinting of complex microbial communities. *Curr Microbiol* 2008;56:553–557.
- Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol* 2008;6:e280.

- 20 Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26:2460–2461.
- 21 Capone KA, Dowd SE, Stamatias GN, Nikolovski J. Diversity of the human skin microbiome early in life. *J Invest Dermatol* 2011;131:2026–2032.
- 22 Dowd SE, Delton Hanson J, Rees E, Wolcott RD, Zischau AM, Sun Y, White J, Smith DM, Kennedy J, Jones CE. Survey of fungi and yeast in polymicrobial infections in chronic wounds. *J Wound Care* 2011;20:40–47.
- 23 Eren AM, Zozaya M, Taylor CM, Dowd SE, Martin DH, Ferris MJ. Exploring the diversity of *Gardnerella vaginalis* in the genitourinary tract microbiota of monogamous couples through subtle nucleotide variation. *PLoS One* 2011;6:e26732.
- 24 Swanson KS, Dowd SE, Suchodolski JS, Middelbos IS, Vester BM, Barry KA, Nelson KE, Torralba M, Henrissat B, Coutinho PM, *et al.* Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. *ISME J* 2011;5:639–649.
- 25 DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 2006;72:5069–5072.
- 26 Zhao J, Carmody LA, Kalikin LM, Li J, Petrosino JF, Schloss PD, Young VB, LiPuma JJ. Impact of enhanced *Staphylococcus* DNA extraction on microbial community measures in cystic fibrosis sputum. *PLoS One* 2012;7:e33127.
- 27 Clarke K, Gorley R. PRIMER v6: User Manual/Tutorial. Lutton, Ivybridge, UK: PRIMER-E; 2006.
- 28 Clarke KR, Somerfield PJ, Gorley RN. Testing of null hypotheses in exploratory community analyses: similarity profiles and biota–environment linkage. *J Exp Mar Biol Ecol* 2008;366:56–69.
- 29 Rogers GB, Cuthbertson L, Hoffman LR, Wing PAC, Pope C, Hooftman DAP, Lilley AK, Oliver A, Carroll MP, Bruce KD, *et al.* Reducing bias in bacterial community analysis of lower respiratory infections. *ISME J* 2013;7:697–706.
- 30 Coenye T, Spilker T, Reik R, Vandamme P, Lipuma JJ. Use of PCR analyses to define the distribution of *Ralstonia* species recovered from patients with cystic fibrosis. *J Clin Microbiol* 2005;43:3463–3466.
- 31 Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, Wolfgang MC, Boucher R, Gilpin DF, McDowell A, *et al.* Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. *Am J Respir Crit Care Med* 2008;177:995–1001.
- 32 Tunney MM, Klem ER, Fodor AA, Gilpin DF, Moriarty TF, McGrath SJ, Muhlebach MS, Boucher RC, Cardwell C, Doering G, *et al.* Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. *Thorax* 2011;66:579–584.
- 33 Rayner MG, Zhang Y, Gorry MC, Chen Y, Post JC, Ehrlich GD. Evidence of bacterial metabolic activity in culture-negative otitis media with effusion. *JAMA* 1998;279:296–299.
- 34 Pillarisetti N, Williamson E, Linnane B, Skoric B, Robertson CF, Robinson P, Massie J, Hall GL, Sly P, Stick S, *et al.*; Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF). Infection, inflammation, and lung function decline in infants with cystic fibrosis. *Am J Respir Crit Care Med* 2011;184:75–81.
- 35 Sly PD, Brennan S, Gange C, de Klerk N, Murray C, Mott L, Stick SM, Robinson PJ, Robertson CF, Ranganathan SC; Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST-CF). Lung disease at diagnosis in infants with cystic fibrosis detected by newborn screening. *Am J Respir Crit Care Med* 2009;180:146–152.
- 36 Zemanick ET, Wagner BD, Harris JK, Wagener JS, Accurso FJ, Sagel SD. Pulmonary exacerbations in cystic fibrosis with negative bacterial cultures. *Pediatr Pulmonol* 2010;45:569–577.
- 37 Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, Hanfland RA, Wohlford-Lenane C, Dohrn CL, Bartlett JA, *et al.* Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Sci Transl Med* 2010;2:29ra31.
- 38 Sun X, Olivier AK, Liang B, Yi Y, Sui H, Evans TI, Zhang Y, Zhou W, Tyler SR, Fisher JT, *et al.* Lung phenotype of juvenile and adult CFTR-knockout ferrets. *Am J Respir Cell Mol Biol* 2014;50:502–512.