The Cystic Fibrosis Airway Microbiome:
*Cultivability, Diversity and Contribution to Disease*

Michael G Surette
Canada Research Chair in Interdisciplinary Microbiome Research
Farncombe Family Digestive Health Research Institute
Departments of Medicine and Biochemistry and Biomedical Sciences
Faculty of Health Sciences, McMaster University, Hamilton ON CANADA
<table>
<thead>
<tr>
<th>Collaborators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvey Rabin MD (Calgary)</td>
</tr>
<tr>
<td>Mike Parkins MD (Calgary)</td>
</tr>
<tr>
<td>Doug Storey (Calgary)</td>
</tr>
<tr>
<td>Cindi Corbett (NML- Winnipeg)</td>
</tr>
<tr>
<td>Scot Dowd (Texas)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Airway Microbiome Team</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tony Schryvers (Calgary)</td>
</tr>
<tr>
<td>Jim Kellner (Calgary)</td>
</tr>
<tr>
<td>Dawn Bowdish (McMaster)</td>
</tr>
<tr>
<td>Jennie Johnstone (McMaster)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Alberta Sepsis Network</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paul Kubes/Chip Doig (Calgary)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Param Nair (McMaster)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chronic Sinusitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martin Derosier (Montreal)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Canadian Cystic Fibrosis Foundation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canadian Institutes of Health Research</td>
</tr>
<tr>
<td>Alberta Heritage Foundation</td>
</tr>
<tr>
<td>Canada Research Chairs</td>
</tr>
</tbody>
</table>

- Matthew Workentine
- Jennifer Stearns
- Anne-Marie Lacroix
- Michelle Pinto
- Michelle Shah
- Chris Sibley
- Margot Grinwis
- Christina Eshaghurshan
- Monica Faria
- Kangmin Duan
- Natalie Marshall
- Jens Norgaard

* Harvey Rabin MD (Calgary)
* Mike Parkins MD (Calgary)
* Doug Storey (Calgary)
* Cindi Corbett (NML- Winnipeg)
* Scot Dowd (Texas)
Cystic Fibrosis

- Autosomal recessive disease caused by mutations in the CFTR chloride ion channel.

- The altered ion transport results in a more viscous pulmonary mucous layer, this impairs ciliary clearance and compromises host immune response permitting microbial colonization of the lung.

- Colonization and establishment of chronic infections ultimately leads to the morbidity and mortality associated with CF.
Cystic fibrosis: organisms isolated from the lower respiratory tract

Data compiled from Cystic Fibrosis Foundation Patient Registry, 2003.
CF Airways are Colonized by Polymicrobial Communities

Example of a molecular profile of the most abundant organisms the CF airway of a single patient.

We and others have shown that the CF airways are colonized by complex microbial communities using mostly culture independent methods.

The thickness represents relative abundance

*K. Bruce, S. Elborn, J.K. Harris, G. Doring, J. Rolain, K. Mathee*

From the vertical dash line, about 65 samples over 1 year (before the dash line, 8 frozen samples going back about 5 years)

The community has stable members but is quite dynamic over time.
The CF Airway Microbiome:

Summary of 25 years of clinical culture data for our patient population

~25,000 isolates / size of circle ~ # of strains
The CF Airway Microbiome:

Data from our lab, ~ 4 years of extensive culturing, about 4000 strains

- Do any of these organisms (other than the tradition CF pathogens) contribute to disease progression?

- And if so does provide new opportunities for management of chronic airway disease?

Most of these are considered normal microbiota from the upper airways.
Simplified View of Disease Progression in CF

Exacerbations are due to an overt immune response to infection and resolution is through aggressive antibiotic treatment.

There are no clinical or microbiological predictors of exacerbation that would allow early intervention.

The cycles of exacerbation result in accumulating loss of lung function over time, often accelerating in frequency towards the end.

Frequently result in an irreversible drop in lung function.

Periods of relative stability interrupted by pulmonary exacerbations.
Simplified View of Disease Progression in CF

We might predict the pathogen load to increase with exacerbation.

Many (> 50%) of adult patients are “non-responders”

The bacterial load of the principal pathogen is the same **before, during** and **after** exacerbations.

How can we explain exacerbations in our adult population?

1. There are pathogens that we have missed that correlate with onset and resolution of exacerbations.

Overlooked Pathogens in CF—The *Streptococcus milleri* group

Accounts for up to 40% of hospitalizations in our adult CF populations

Managing SMG in Adult CF

- homozygous ΔF508 cftr
- Chronic *P. aeruginosa*
- Prophylactic and directed SMG antibiotic therapy
- Reduced hospitalizations
- Stabilization of FEV₁

Sibley et al 2009 Curr Opin Investig Drugs
Pulmonary Exacerbations in CF

40% *Streptococcus* Milleri Group
15% *Gemella haemolysans* and other spp

Adult CF Exacerbations

Polymicrobial interactions with primary pathogen *P. aeruginosa*
- a role for the *avirulent* oropharyngeal flora?

We had previous studies in rats to support this model Duan et al 2003
Simple Infection System for Polymicrobial Infections

Polymicrobial Infections are Complex

- different SYNERGENS (Class III infections) “behave” the same (enhanced host death)
- from the fly’s perspective (innate immunity / AMP expression) these two Class III infections are very different
- from the *P. aeruginosa* perspective (virulence gene expression) these two Class III infections are very different

See Sibley et al PLoS Path 2008 for details
Class III - SYNERGENS
avirulent – but activate *P. aeruginosa*

- Over 1/3 of the isolates tested fell into this class
- Do not breakdown by genus or species
Making sense of the host response in chronic infections like CF is complicated by incomplete microbiology.

What organisms are contributing to disease is not so easily defined and can not be based on their pathogenicity in isolation.

- More potential microbes to target with current antibiotics
- More potential pathways to develop new approaches

Synergistic infections ≠ Cooperative behavior
The CF Airway Microbiome: *Cultivatable vs Uncultivatable Organisms*

This collection of strains includes many not previously cultured – so the question we decided to ask was- what is the make of this community with respect to Cultivatable vs Uncultivatable Organisms?

i.e. how good are we if we really try to culture ....
Culture-enriched molecular profiling of the CF airway microbiome

*Cultivatable vs uncultivatable microbes*

Sputum sample

~ 24 culture conditions (half were anaerobic)

Molecular profile
T-RFLP/deep sequencing

24 different profiles

Sibley et al submitted
Culture Enriched Profiles
(by condition)

Colistin Nalidixic Acid Blood Agar

5% CO₂

Anaerobic

12 peaks/species

4 peaks/species

% Total Peak Area

T-RF Size (bp)
T-RFLP Profile of sputum sample

- each peak represents at least one different bacterial species

- 13 peaks in this sample

- equivalent to $10^3$-$10^4$ 16sRNA sequences
For each peak observed in the sputum sample, we can look for conditions where the peak is found in the culture enrichment.
When we look at this data for all peaks that we can culture, we see that almost half are obligate anaerobes (we see similar results with most airway samples).
Each Patient has a unique CF Airway Microbiome

- Each patient has a different complement of culture conditions required to cultivate the airway microbiome.
Using the T-RFLP as a guide we did 454 sequencing on 4 patients

• 16S PCR and sequencing with Flx Titanium

• used only read lengths > 400 nt

• for each sputum sample we generated about 200,000 reads

• for 40 different culture-enriched samples we generated ~ 4000 reads each

• a total of > 1 million usable 16S sequence reads
The uncultured 5 Families represent very rare organisms represented by singletons or doubletons in our deep sequencing (i.e. 1 or 2 out of ~200,000 16S sequences).

We identified 4 other families by culture that we did not get by deep sequencing.

At the Family and Genera level, we are able to culture ~90% identified groups identified by deep sequencing.
Three simple take-home messages:

1. Most airway infections will be polymicrobial and may include **overlooked pathogens** (not just a CF story)

2. but perhaps more commonly **SYNERGENS** – otherwise benign or even beneficial organisms that increase disease in the presence of a pathogen.

3. The great majority of the airway microbiome (and likely all of the human microbiome) is cultivatable without the need to develop new culture media.
Some lessons learned:

The CF patient population is not uniform and comparing these patients as a group to controls is not necessarily the most informative approach.

In our studies of adult CF patients over the last several years, multiple mechanisms of disease progression are observed:

• The dynamics of conventional CF pathogens (e.g. P. aeruginosa) correlate with disease in some patients
• There are overlooked pathogens missed by conventional CF microbiology such as the Streptococcus milleri group (and other more unusual organisms in rare cases)
• There are likely synergens driving disease in many patients where there is no change in the dynamics of conventional CF pathogens.
• other mechanisms ...
Some lessons learned:

As we all know DNA extraction is critical

- *We have benchmarked our methods against culture data – making sure organisms we knew we abundant are well represented in the molecular signature.*

Our protocol (1) compared to two common methods for DNA Extraction from Stool (DGGE Profile)

- *Our experience has been that it is easy to miss even abundant organisms without some method to validate on real samples.*
Some lessons learned:

**DNA vs viable cells**

*How does the molecular signature match up with culture?*

- K. Bruce’s group – *P. aeruginosa* CFU not reflected in extracted DNA levels
- Our group – for *Streptococcus milleri* about - a 3 day lag in molecular signature to CFU

The “stability“ of DNA differs between organisms – we know the bacteria living in the airways are in microaggregates/biofilms (probably other sites)

*P. aeruginosa* incorporates its DNA into the biofilm matrix

*Streptococcus constellatus*

- wt
- DNAse mutant

Other bacteria produce active DNAses

Courtesy of Shawn Lewenza (Calgary)
Some final food for thought...

48 Families found in our CF sputum studies to date

<table>
<thead>
<tr>
<th>Actinomycetaceae</th>
<th>Dermabacteraceae</th>
<th>Peptostreptococcaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerococcaceae</td>
<td>Enterobacteriaceae</td>
<td>Planococcaceae</td>
</tr>
<tr>
<td>Bacillaceae</td>
<td>Enterococccaceae</td>
<td>Porphyromonadaceae</td>
</tr>
<tr>
<td>Bacillales Incertae Sedis</td>
<td>Erysipelotrichaceae</td>
<td>Prevotellaceae</td>
</tr>
<tr>
<td>Bacteriovoracaceae</td>
<td>Eubacteriaceae</td>
<td>Propionibacteriaceae</td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>Flavobacteriaceae</td>
<td>Pseudomonadaceae</td>
</tr>
<tr>
<td>Bifidobacteriaceae</td>
<td>Fusobacteriaceae</td>
<td>Rhizobiaceae</td>
</tr>
<tr>
<td>Burkholderiaceae</td>
<td>Lachnospiraceae</td>
<td>Rhodospirillaceae</td>
</tr>
<tr>
<td>Campylobacteraceae</td>
<td>Lactobacillaceae</td>
<td>Rikenellaceae</td>
</tr>
<tr>
<td>Carnobacteriaceae</td>
<td>Micrococcaceae</td>
<td>Ruminococcaceae</td>
</tr>
<tr>
<td>Clostridiaceae</td>
<td>Moraxellaceae</td>
<td>Spirochaetaceae</td>
</tr>
<tr>
<td>Clostridiales Family XI.</td>
<td>Mycobacteriaceae1</td>
<td>Staphylococcaceae</td>
</tr>
<tr>
<td>Clostridiales Family XIII.</td>
<td>Mycoplasmataceae</td>
<td></td>
</tr>
<tr>
<td>Comamonadaceae</td>
<td>Neisseriaceae</td>
<td>Streptococcaceae</td>
</tr>
<tr>
<td>Coriobacteriaceae</td>
<td>Nocardiaaceae</td>
<td>Thermotogaceae</td>
</tr>
<tr>
<td>Corynebacteriaceae</td>
<td>Pasteurellaceae</td>
<td>Veillonellaceae</td>
</tr>
</tbody>
</table>

Still very much an oversimplified view
Complexity at the species level

Distribution of Streptococci Isolated from CF Sputum

- pyogenic group
- anginosus group
- mutans group
- salivarius group
- mitis group

% cultured Streptococcaceae

12.8 ✔ 1

n = 993

novel species
Complexity at the strain level:
Phenotypic heterogeneity in *P. aeruginosa*
Multiple isolates form a single patient in a single sample

All derived from a single colonizing strain
Complexity at the strain level:

Variability in two acute virulence phenotypes between and within distinct morphotypes from 1 patient

Profiles for 24 different phenotypes....

The same heterogeneity is observed in antibiotic resistance profiles.