KING'S College LONDON

Anaerobes in sinusitis and chronic lower respiratory tract infections

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ECCMID Tuesday May 13, 2014

Structure for today

Thanks for the invitation to talk

Chronic lower respiratory infections

Sinus infections to cover

- Millions affected by these conditions
- Clearly important to understand better

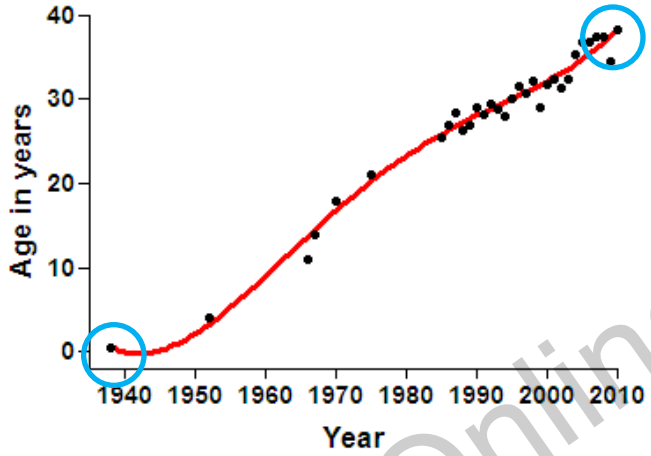
This talk summary works through

- Initial infection “model”
- Methodologies – two ways; two-way
- Detection of anaerobes
- Persistence of anaerobes
- Significance of anaerobes
- Conclusions

Many chronic lower respiratory infections – cystic fibrosis as the first model.

Cystic fibrosis – a chronic airways infection

Extending the gains in CF life expectancy is a key issue



Median predicted mortality age from 6 months (1938) to ~40 years (2010)

Cystic Fibrosis Foundation Patient Registry Data, 2010/ John LiPuma

The cause of death in CF though usually is "***respiratory failure brought on by chronic bacterial infection and concomitant airway inflammation***" Lyczak *et al.* (2002)

<u>Key points</u>

- Importance of antibiotics in maintaining respiratory health
- Tackling microbial infections slows chronic disease progression
- In turn, important to know which species are present.

Species causing CF airways infection

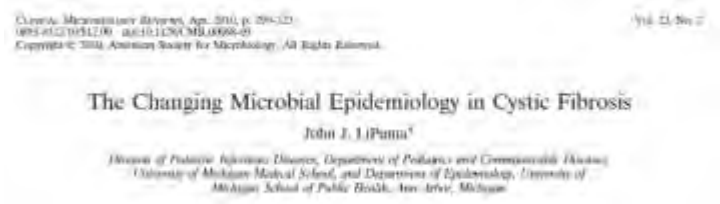
How well do we know the species present in the airways?

CF species often detected by culture

- *Burkholderia cepacia* complex
- *Haemophilus influenzae*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Stenotrophomonas maltophilia*

These together with certain fungi e.g. *Aspergillus fumigatus* have been considered key CF pathogens

see also



The Changing Microbial Epidemiology in Cystic Fibrosis

John J. LiPuma

With Tony Hart here in 1999, our original **working hypothesis** was that any species may be potentially important in lung function decline.

Rationale

Traditionally, detection from airway secretions has relied on *in vitro* **growth** on selective media under defined, mostly aerobic, conditions - important and features heavily today

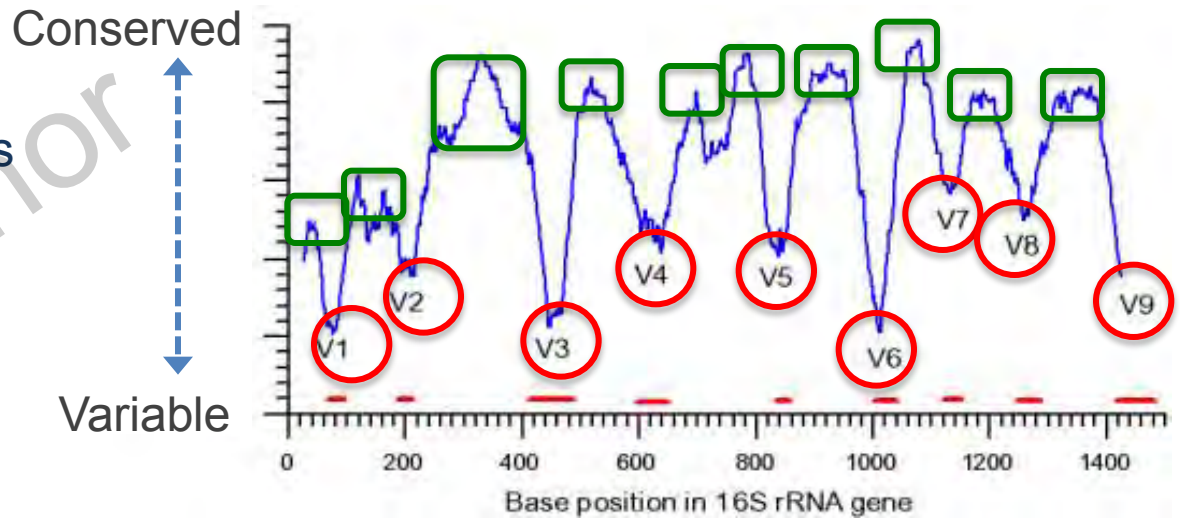
Were any bacterial species missed though?

To ask that, we used a way of studying bacteria **without culture**

"*It sounds too good to be true: Take water from the ocean or from deep underground, find the DNA in it, sequence the genes, and use them to identify the organisms that live there*" Science, 2004

Either sequence all DNA (metagenomics) or ribosomal gene regions to identify species.

As before, this schematic shows that 16S rRNA gene sequences contain **hypervariable** regions and **conserved** regions.

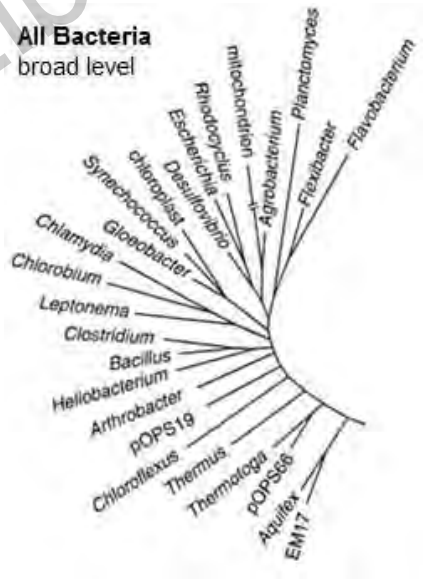


Methodology

Collect samples from airways, sinus, other

Extract nucleic acids

Amplify ribosomal gene regions – broad range PCR
Patchwork of highly variable and conserved regions
- –Conserved regions serve as primers e.g. for “all” Bacteria
- –Highly variable regions identify single species
- –One sequence identifies each bacterial species present



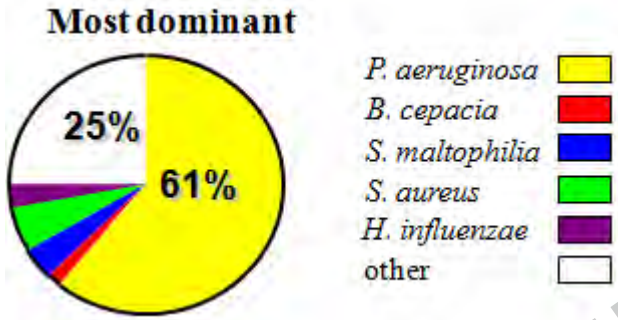
Sequence
- – database comparisons to identify species

Analyse data – for today, which genera/ species are present?
- – species number, diversity etc, to define composition, clinical metadata

This methodology was applied to sputum sampled from 34 adults with CF
- we first asked what bacterial species were common.

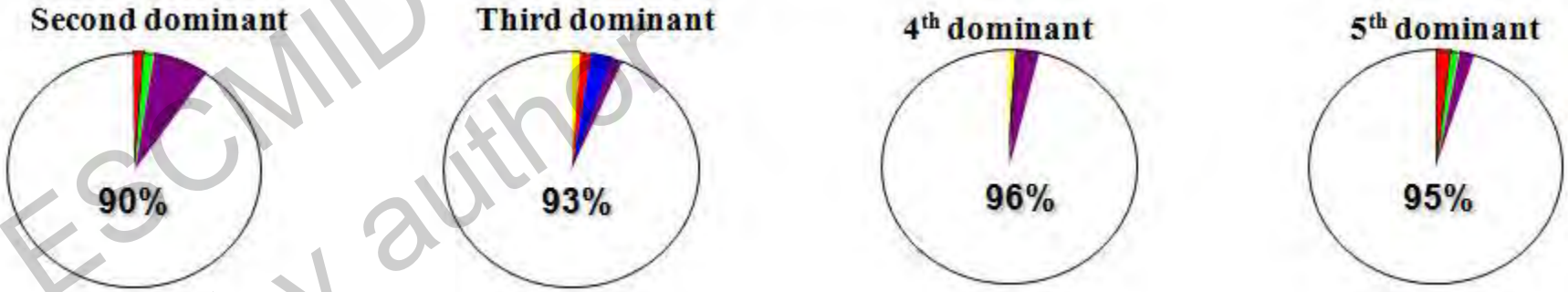
Each sample contained a diverse mix of species

P. aeruginosa was the most abundant species in 21 of 34 of the sputum-productive adults (Rogers *et al*., 2004)



A quarter of patients then had a non-"key pathogen" as the most abundant species

Other species were more common still as the 2nd-5th most abundant species



So, what were these other species?

Anaerobes in CF respiratory samples

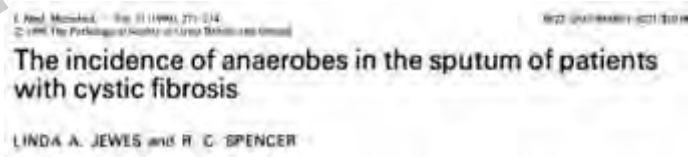
Though now tiny, a set of data from a decade ago makes important points clearly

- Three CF patients, 53 clone identities determined 19 species

Detected species included

- Potential **pathogens** in other contexts - *Bacteriodes gracilis*
- Many species **novel to CF airways** e.g. *Abiotrophia adiacens*
- Many **obligate anaerobes**

Anaerobes had been reported before in CF sputa here in 1990


The incidence of anaerobes in the sputum of patients with cystic fibrosis

LINDA A. JEWES and R. C. SPENCER

Also, Brook and Fink (1983)

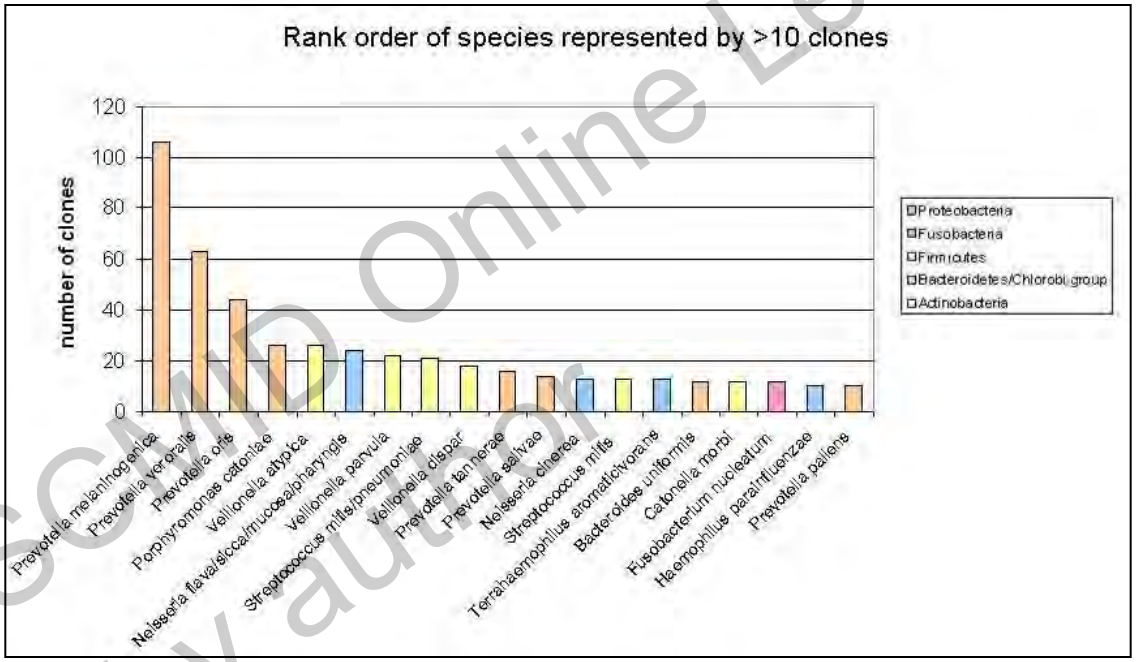
"*The recovery of anaerobic organisms from four of the six transtracheal aspirations specimens suggests a possible role for these organisms in the etiology of pulmonary infection in cystic fibrosis."*

This prompted a more detailed analysis.

Anaerobes in CF respiratory samples – detail

Sequence identities from a fresh study of 2139 high quality ribosomal gene clones as generated from fourteen sputum productive CF patient

- *P. aeruginosa* was again common
- Excluding *P. aeruginosa* reveals the abundance of anaerobic species



Prevotella, *Veillonella*, *Porphyromonas* all common genera- 1st 5 here

Oral cavity / upper airway origin of many anaerobes implied

Supporting work – anaerobic areas in infected CF airways

Was there though any evidence that anaerobic conditions occur in the airways?

Yes:

Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients

Dieter Worlitzsch,[1] Robert Tarran,[2] Martina Ulrich,[1] Ute Schwab,[2] Aynur Cekici,[1] Keith C. Meyer,[3] Peter Birrer,[4] Gabriel Bellon,[5] Jürgen Berger,[6] Tilo Weiss,[7] Konrad Botzenhart,[1] James R. Yankaskas,[2] Scott Randell,[2] Richard C. Boucher,[2] and Gerd Döring[1]

Developmental Cell, Vol. 3, 593–603, October, 2002, Copyright ©2002 by Cell Press

***Pseudomonas aeruginosa* Anaerobic Respiration in Biofilms: Relationships to Cystic Fibrosis Pathogenesis**

- "in CF patients with established lung disease, *P. aeruginosa* was located within hypoxic mucopurulent masses"
- "*P. aeruginosa* infection ... reflects biofilm formation and persistence in an anaerobic environment"

Given the oral cavity / upper respiratory origin of many anaerobic species detected, were these merely contaminants at time of sampling?

Supporting work – addressing contamination and dead cells

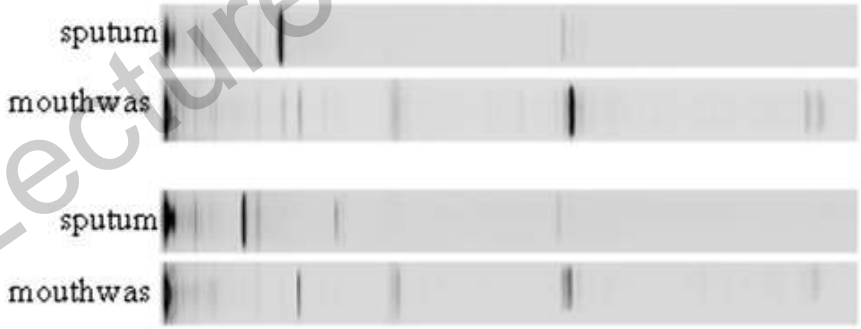
Anaerobes as sampling contaminants?

- Sputum transits the upper respiratory tract/ oral cavity during expectoration

However,

- Paired mouthwash and sputum samples from same adult with CF were distinct (Rogers *et al*., 2006)

Return to sampling later

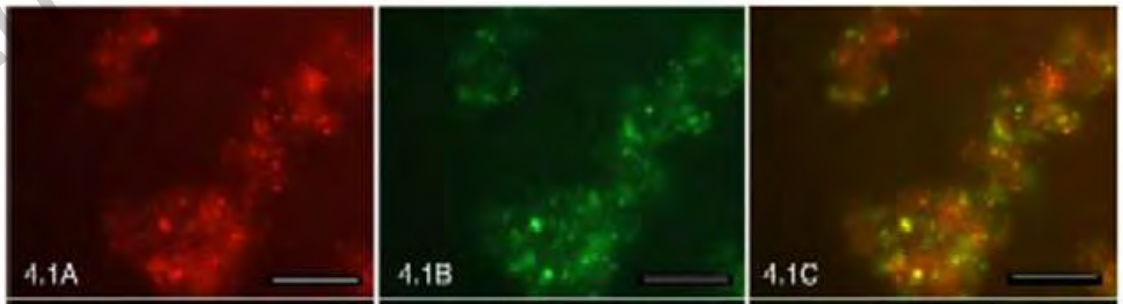


These microbes will be exposed to antibiotics and host immune response

- **Were we just looking at dead cell signals from anaerobes/ wider then?**

CF sputum sample - red = dead cells green = viable cells composite

Propidium based live dead staining



PMA treatment of sputum

A similar propidium-based chemistry was applied to exclude dead bacteria from detection by PCR

Propidium monoazide (PMA):

- Enters only dead cells
- Cross-links DNA in those and extracellularly
- Only live cells are detected by PCR

See also Fittipaldi *et al*. (2009)

In another CF sputum study,

- PMA was used in this study of 386 213 ribosomal sequences from 30 CF samples

Classified species as either common or scarce

Three anaerobic genera as before – species detected in the 30 sputa

Species name	Non-PMA treatment
Prevotella denticola	C
Prevotella histicola	C
Prevotella melaninogenica	C
Prevotella nanceiensis	C
Prevotella oris	S
Prevotella oulorum	C
Prevotella pallens	C
Prevotella ruminicola	S
Prevotella salivae	C
Prevotella verorallis	C
Veillonella atypica	C
Veillonella dispar	C
Veillonella parvula	C
Porphyromonas bennonis	C
Porphyromonas catoniae	S

- Without PMA treatment, twelve species were regarded statistically as common (C) and three scarce (S)
- If signals were just from dead cells these should be removed by PMA treatment.

Evidence for viable anaerobic cells in CF sputum

Species name	Non-PMA treatment	PMA treatment
Prevotella copri		S
Prevotella denticola	C	C
Prevotella histicola	C	S
Prevotella melaninogenica	C	C
Prevotella nanceiensis	C	C
Prevotella oris	S	S
Prevotella oulorum	C	S
Prevotella pallens	C	C
Prevotella ruminicola	S	
Prevotella salivae	C	C
Prevotella verorallis	C	S
Veillonella atypica	C	C
Veillonella dispar	C	C
Veillonella parvula	C	C
Porphyromonas bennonis	C	
Porphyromonas catoniae	S	S

- The signal from eleven species stayed the same or increased post PMA
- These results suggest anaerobes exist as viable cells in the airways.

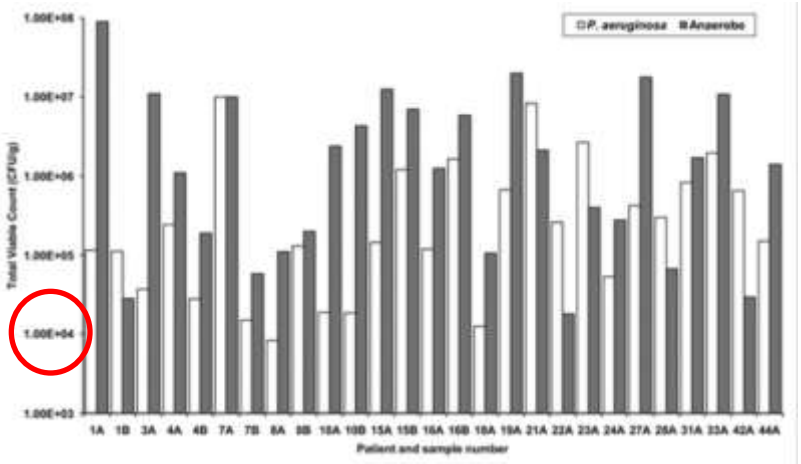
Strong support for this came from culture-based studies

Detection of Anaerobic Bacteria in High Numbers in Sputum from Patients with Cystic Fibrosis

Michael M. Tunney[1], Tyler R. Field[1], Thomas F. Moriarty[1], Sheila Patrick[2], Gerd Doering[3], Marianne S. Muhlebach[4], Matthew C. Wolfgang[5,6], Richard Boucher[6,7], Deirdre F. Gilpin[1], Andrew McDowell[2], and J. Stuart Elborn[2]

- 2008: Concordance between culture and sequence-based methods

- Species of the genera *Prevotella* and *Veillonella* detected in sputa from 18 of 50 adults with CF
- Wider, anaerobes were abundant (>10^4 cfu/g) in the majority of sputum samples
  - Higher than expected if contamination
  - Often as abundant, or more abundant, than the species considered pathogens
  - More often cultured from samples with *P. aeruginosa* than those without it ($p<0.01$)



So, anaerobes are present, but do they persist in these chronic infections?

Longitudinal analysis determines persistence

A snapshot of CF sputum microbiota over 300 days

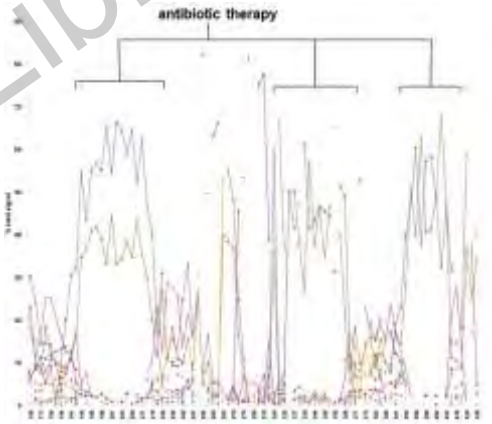
x axis – days, y axis - % total signal

Each colour signifies one species

Proportions of each species change over the 45 sputa here

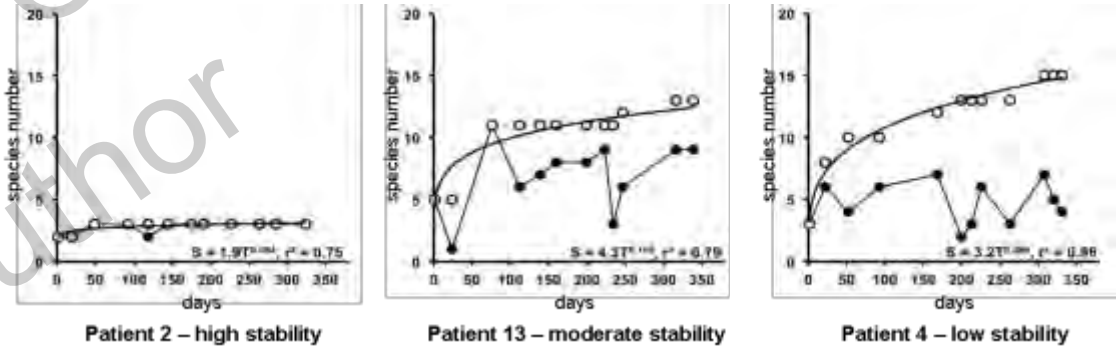
Antibiotic impacts

Can be highly dynamic therefore



Simplifying this, cumulative species richness – here open circle plots - monthly over a year could show no change in species (a flat line) over time or new species being detected constantly (a rising line) as outcome "extremes"

Stressmann, 2012



Patient 2 – high stability | Patient 13 – moderate stability | Patient 4 – low stability

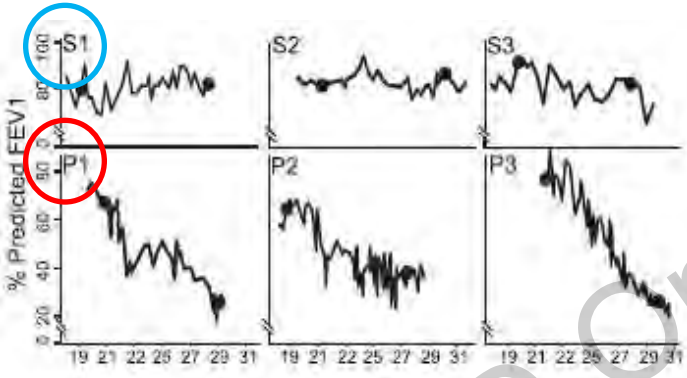
For the majority of patients, the species present at the start of the year persisted.

- Including anaerobes.

But the rates of addition of new species varied between patients.

Anaerobes persist in the CF airways

Zhao *et al*. (2012) also looked at persistence* in six patients whose lung disease was either Progressing (P) or Stable (S) over ~ a decade



PNAS

Decade-long bacterial community dynamics in cystic fibrosis airways

Jingchao Zhao[a], Patrick D. Schloss[b], Linda M. Kalikin[a], Lisa A. Carmody[a], Bridget R. Foster[a], Joseph F. Petrosino[c,d], James D. Cavalcoli[e], Donald R. VanDevanter[f], Susan Murray[g], Jun Z. Li[h], Vincent B. Young[b,i], and John J. LiPuma[a,1]

Department of [a]Pediatrics and Communicable Disease, [b]Microbiology and Immunology, [c]Human Genetics, and [d]Internal Medicine/Infectious Disease Division and [h]Center for Computational Medicine and Bioinformatics, University of Michigan Medical School, Ann Arbor, MI 48109; [c]Alkek Center for Metagenomics and Microbiome Research and [g]Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX 77030; [e]Department of Pediatrics, Case Western Reserve University, Cleveland, OH 44106; and Departments of [f]Biostatistics and [i]Epidemiology, University of Michigan School of Public Health, Ann Arbor, MI 48109

Edited by James M. Tiedje, Michigan State University, East Lansing, MI, and approved February 17, 2012 (received for review September 19, 2011)

Table S3. Average relative abundance (%) of persistent OTUs

S1	S2	S3	P1	P2	P3
All samples					
Pseudomonas-1* (75.0)	*Pseudomonas*-1 (75.2)	*Pseudomonas*-1 (34.3)	*Pseudomonas*-1 (39.2)	*Pseudomonas*-1 (46.6)	*Pseudomonas*-1 (61.2)
Streptococcus-1* (5.3)	*Streptococcus*-1 (3.4)	*Streptococcus*-1 (11.8)	*Streptococcus*-1 (10.3)	*Streptococcus*-1 (6.1)	*Streptococcus*-2 (5.7)
Streptococcus-2* (7.0)	*Streptococcus*-2 (7.2)	*Streptococcus*-2 (8.3)	*Streptococcus*-2 (5.7)	*Streptococcus*-2 (7.0)	*Staphylococcus* (16.0)
	Veillonella (4.0)	*Veillonella* (2.1)	*Veillonella* (3.3)	*Veillonella* (1.2)	
		Prevotella-1 (2.5)	*Prevotella*-1 (3.5)	*Prevotella*-1 (3.3)	
		Fusobacterium (13.7)	*Actinomyces* (23.2)	*Staphylococcus* (18.7)	
		Porphyromonas-1 (6.6)			
		Rothia (5.4)			

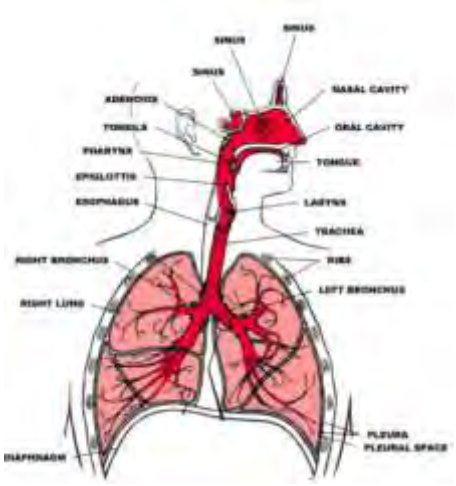
* Persistent - a relative abundance of >1% in over 50% of the samples from each individual over the nine years of study

Found

- Persistence of *Veillonella*, *Prevotella* and *Porphyromonas* genera for a decade
- Even in the presence of *Pseudomonas*
- In both stable and progressing patients

Are anaerobes in other chronic respiratory infections & sinusitis though?

Hypothesis: Anaerobes will be present in other chronic airway infections



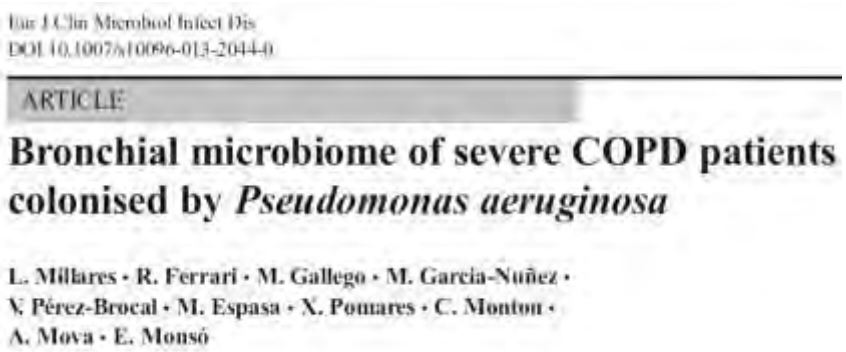
- The sinuses, oral cavity and lower respiratory tract are **continuous**
- The same species could be important in both chronic upper and lower airway infections
  - Including COPD, non-CF bronchiectasis, chronic sinusitis

Some evidence for this exists in studies on *P. aeruginosa* in CF:

- Ciofu *et al*. (2013) “Bilateral **exchange** of *P. aeruginosa* isolates between the paranasal **sinuses and** the **lungs** occurs in chronically infected patients”

Time to highlight only excerpts from selected COPD, non-CF bronchiectasis and chronic sinusitis studies.

Chronic obstructive pulmonary disease (COPD) and anaerobes

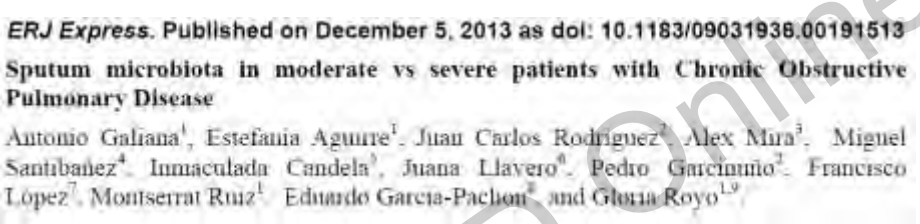


Eur J Clin Microbiol Infect Dis
DOI 10.1007/s10096-013-2044-0

ARTICLE

Bronchial microbiome of severe COPD patients colonised by *Pseudomonas aeruginosa*

L. Millares · R. Ferrari · M. Gallego · M. Garcia-Nuñez · V. Pérez-Brocal · M. Espasa · X. Pomares · C. Monton · A. Moya · E. Monsó



ERJ Express. Published on December 5, 2013 as doi: 10.1183/09031936.00191513

Sputum microbiota in moderate vs severe patients with Chronic Obstructive Pulmonary Disease

Antonio Galiana[1], Estefania Aguirre[1], Juan Carlos Rodriguez[2], Alex Mira[3], Miguel Santibañez[4], Inmaculada Candela[5], Juana Llavero[6], Pedro Garcinuño[7], Francisco López[7], Montserrat Ruiz[1], Eduardo Garcia-Pachon[8], and Gloria Royo[1,9]

Outgrowth of the Bacterial Airway Microbiome after Rhinovirus Exacerbation of Chronic Obstructive Pulmonary Disease

Philip L. Molyneaux[1,2], Patrick Mallia[1,2,3], Michael J. Cox[1,2], Joseph Footitt[1,2,3], Salfron A. G. Willis-Owen[1], Daniel Homola[1], Maria-Belen Trujillo-Torralbo[1,2,3], Sarah Elkin[1,2], Onn Min Kon[1,2,3], William O. C. Cookson[1,3], Miriam F. Moffatt[1,2], and Sebastian L. Johnston[1,2,3]

Parallels in non CF bronchiectasis?

Millares "*Veillonella*, *Actinomyces*, *Granulicatella*, *Neisseria*, *Prevotella*, *Tannerella*, *Gemella*, *Rothia* and *Achromobacter*, with *Streptococcus* most abundant"

Galiana "a common bacterial core consisting of the genera *Rothia, Prevotella, Veillonella, Fusobacterium, Porphyromonas* and *Haemophilus*"

Molyneaux "*Streptococcus* was the most common genus (42.5% of total reads) followed by *Veillonella* (15.2%) and *Prevotella* (15.0%)" in mild COPD.

Non-CF bronchiectasis and anaerobes

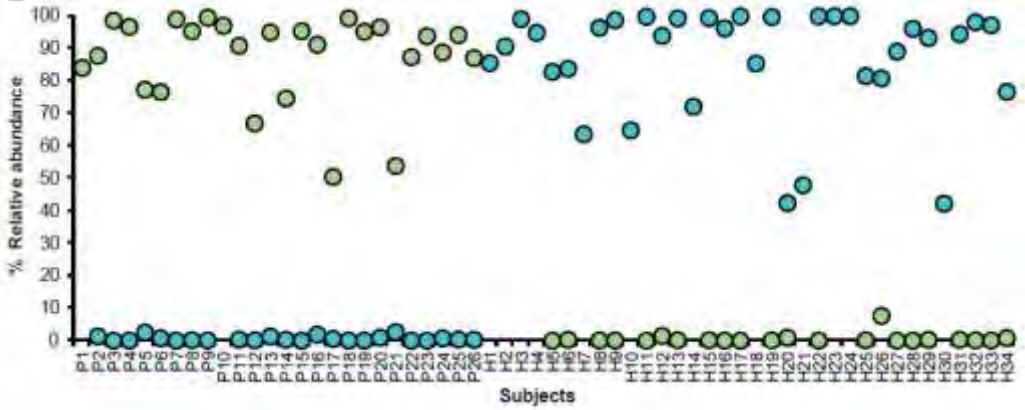
Lung Microbiota and Bacterial Abundance in Patients with Bronchiectasis when Clinically Stable and during Exacerbation

Michael M. Tunney[1,2*], Gisli G. Einarsson[1,2*], Lan Wei[1,3], Maire Drain[1], Erich R. Klem[4], Chris Cardwell[5], Madeleine Ennis[5], Richard C. Boucher[4], Matthew C. Wolfgang[3,4], and J. Stuart Elborn[1,2*]

In sputum from 21 individuals, *Veillonella* and *Prevotella* common by culture-dependent and –independent methods.

Genus*	Total Sequences	Relative Abundance (%) of total OTU's
Haemophilus	28497	30.71
Streptococcus	13745	14.81
Pseudomonas	11699	12.61
Bacteria;Unclassified	8288	8.93
Achromobacter	8164	8.80
Veillonella	5631	6.07
Prevotella	3998	4.31
Stenotrophomonas	2683	2.89

In a new study, samples from 60 adults with bronchiectasis were either dominated by *P. aeruginosa* (green, *n* = 26) or *Haemophilus influenzae* (blue, *n* = 34)



- *Prevotella* spp. were more abundant in *P. aeruginosa*-dominated samples ($P < 0.0001$)
- *Leptotrichia* spp. were more abundant in *H.influenzae* dominated samples ($P < 0.0001$)

Unpublished data, pers comm Chris van der Gast

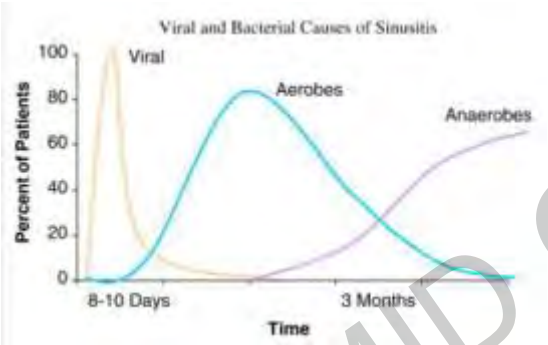
Do these associations between species indicate interactions?

Chronic rhinosinusitis (CRS) and anaerobes

Bacteriology of Chronic Sinusitis and Acute Exacerbation of Chronic Sinusitis

Itzhak Brook, MD, MSc

Culture of chronic sinus infections identified a range of anaerobes



Brook (2010) suggested that sinus infections become increasingly anaerobe dominated

Table. Organisms Isolated From Patients With Chronic Maxillary Sinusitis or Maxillary Acute Exacerbation of Chronic Sinusitis (AECS)*

Bacteria	Patients With Chronic Sinusitis (n = 32)	Patients With AECS (n = 30)
Anaerobic bacteria		
Peptostreptococcus species	16	14
Propionibacterium acnes	3	4
Fusobacterium species	3 (1)	4 (3)
Fusobacterium nucleatum	5 (1)	6 (4)
Bacteroides species	2	3 (2)
Prevotella melaninogenica	4 (1)	6 (5)
Prevotella intermedia	6 (2)	4 (3)
Prevotella oralis	4	5 (1)
Porphyromonas asaccharolytica	5 (1)	3 (2)

- Stressmann *et al*. (2012) detected obligate anaerobes including *Prevotella*
- Boase *et al*. (2013) reported anaerobes but not obligate species in CRS
- Immune response may be important in shaping microbiota

Original Investigation

Contrasting the Microbiomes From Healthy Volunteers and Patients With Chronic Rhinosinusitis

Though *Prevotella* spp. seem common, additional work is needed to form a consensus in CRS.

Significance of anaerobes? Caveats

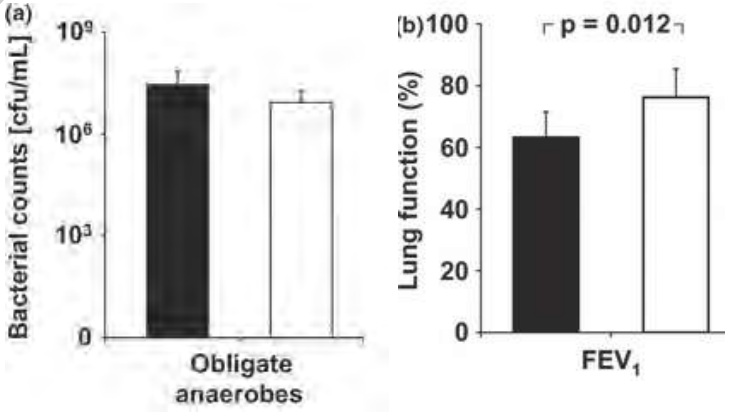
Anaerobes can be detected in the airways.

- What is their role in disease pathophysiology?

Three caveats:

- An impact by a species on pathophysiology in one condition does not necessarily mean the same follows for another condition.

- Worlitzsch *et al*. (2009) showed no change in obligate anaerobe abundance
  - With antibiotics for CF exacerbation
  - Despite clinical improvement



- In COPD and healthy individuals "a common core microbial community was identified that included members of *Acinetobacter, Fusobacterium, Megasphaera*, *Prevotella*, *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptococcus,* and *Veillonella*" (Zakharkina *et al*., 2013)

Perhaps anaerobes are opportunistic pathogens at specific phases of infection?

Significance of anaerobes?

Understanding the roles of anaerobes in chronic disease is complicated by:

- Sampling difficulties and analytical methodology variation
- Variation in the anaerobes detected between individuals
- Variation in anaerobes detected at different stages of chronic infection

- The range of anaerobic species reported (e.g. in CF, Su and Hassett, 2012) is likely to rise as sequencing power increases

So, which genus and/ or species to focus on first then?

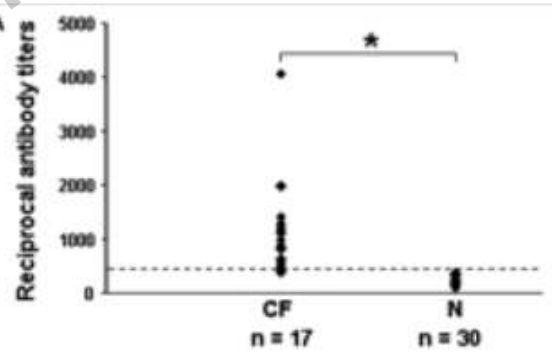
- *Prevotella* spp. are frequently the cause of infections at many body locations (Jousimies-Somer *et al*., 2003)
  - These are a clear target of research (Field *et al*., 2010)

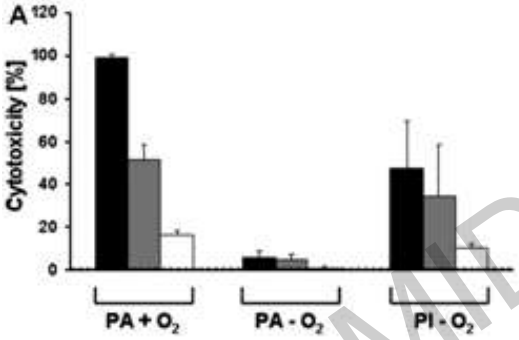
Significance of *Prevotella*?

Ulrich *et al*.(2010) studied the frequently detected species *Prevotella intermedia*

Bronchoscopy identified *Prevotella* species >10^5 CFU/g - Tunney *et al*. (2008)

Most CF patients but not healthy controls had a measureable antibody titre against *Prevotella intermedia* (p<0.001)





P. intermedia produced more cytotoxins than *P. aeruginosa* when grown anaerobically

- Specific obligate anaerobes therefore may be acting as pathogens in a context dependent manner
- *Prevotella* can also produce beta-lactamases (Field *et al*., 2010) – so even if not acting as a pathogen, this can impact on infection progression through indirect **interaction.**

Interactions

Many anaerobe cells are present in airways samples

- These are likely to interact with both the host or other microbes

Two examples of differential consequences of interaction

- First from 2008 on infection outcome:

Human oropharyngeal bacteria can dramatically enhance the ability of *Pseudomonas aeruginosa* to kill flies.

OPEN ACCESS Freely available online PLoS PATHOGENS

Discerning the Complexity of Community Interactions Using a *Drosophila* Model of Polymicrobial Infections

Christopher D. Sibley[1], Kangmin Duan[1,2], Carrie Fischer[1], Michael D. Parkins[3,4], Douglas G. Storey[1,5], Harvey R. Rabin[1,3,4], Michael G. Surette[1,6]*

"a large proportion of the organisms in CF airways has the ability to influence the outcome of an infection when in combination with the principal CF pathogen Pseudomonas aeruginosa*."*

This also supported by a report from 1994

J. Med. Microbiol. — Vol. 40 (1994), 118–123

A mechanism of pathogenicity of "*Streptococcus milleri* group" in pulmonary infection: synergy with an anaerobe

T. SHINZATO and A. SAITO

Inoculum*	Histological findings		Mortality†
	Pneumonia	Lung abscess	
S. constellatus	Mild, improved by 7 days	None	10%
P. intermedia	Mild, improved by 7 days	None	10%
S. constellatus + *P. intermedia*	Severe, continued for 14 days	60% in 8–14 days	60%‡

†n = 10 in each group.
‡$p < 0.05$ compared with single infection by *S. constellatus* or *P. intermedia*.

Conclusions

- Many obligate anaerobes are present in high numbers in chronic sinus and airways infections

- *Prevotella*, *Veillonella* and *Porphyromonas* are common
  - Also present in healthy airways
  - May be pathogens in specific contexts, :
    - Individually
    - In combination with other species

- Evidence for persistence over a decade in the same chronic infection

Ways forward?

- Extending animal models and human subject work
- Relating gene expression of anaerobes to the host/ outcome
- Defining effects of targeted treatment

More work needed therefore to understand the true nature of these companions.

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