Molecular diagnostics in cystic fibrosis microbiology

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CF microbiology

• Key concepts
  – Phenotypic adaptations to CF airway
  – Polymicrobial infections
  – Viral, bacterial, mycobacterial and fungal infections may co-exist
Topics for today

- Identification of mucoid *P. aeruginosa*
- Identification of other non-fermenting gram-negative rods (NFGNRs)
- Viral detection in CF sputum
- Detection of “non-culturable” organisms
How do we identify NFGNRs?

• Basic phenotype
  – Colonial appearance, unique features (mucoidy, pigmentation)
  – Smell of colonies on plate
  – Gram staining
How do we identify NFGNRs?

• Biochemical testing
  – Limited # of tests
  – Clear endpoints
  – NOT kits or automated methods

• Quickly go to PCR
  – Amplify specific genes
    OR
  – Perform PCR sequencing

Pseudo-P, OF-gluc, TSI, Arg, Beta hemolysis
Inaccuracy of rapid testing

• Kiska, 1996: examined the utility of rapid testing for 150 NFGNRs from CF pts.
  – 4 systems tested: RapID NF Plus, Remel, API Rapid NFT, Vitek Auto
  – Accuracy ranged from 57 to 80% overall
  – From 43-86% for *B. cepacia* complex
  – Best performance was for *S. maltophilia*
    • Range 93 to 100% correct
Inaccuracy of rapid testing

• Compared Vitek ID with V3 sequencing
  – 17 non-*P. aeruginosa* isolates compared
  – Vitek IDs included:
    • 1 *A. xylosoxidans*
    • 2 *S. maltophilia*
    • 3 *Acinetobacter* spp. (*A. baumanii*, *A. haemolyticus*, *A. twoffi*)
    • 1 *Achromobacter denitrificans*
    • 1 *P. fluorescens*
    • 1 *Sphingomonas paucimobilis*
    • 2 *Wautersia pacula*
    • 1 *Deftia acidovorans*
    • 1 *Salmonella* ser. *Gallinarium*
    • 4 unidentified organisms
Inaccuracy of rapid testing

- V3 identifications:
  - 7 A. xylosoxidans
  - 1 S. maltophilia
  - 1 Acinetobacter spp.
  - 8 Pseudomonas spp. not aeruginosa

- Only 2 of the Vitek IDs agreed with sequencing:
  - A. xylosoxidans
  - P. fluorescens = Pseudomonas spp. not aeruginosa
Problems with phenotypic ID

- Phenotypic adaptation to CF airway
- Data from RK Ernst (2003), EE Smith (2006), Mena (2008)
  - ↑ Mucoidy
  - ↓ Motility and other virulence factors
  - Acquisition of lasR mutations
  - Hypermutable phenotype
- Causes:
  - Antibiotics, other organisms, biofilm formation
Spectrum of difficulty

- Mucoid *P. aeruginosa*
- “Big 4” Gram negative rods
  - Non-mucoid *P. aeruginosa*
  - *Burkholderia cepacia* complex
  - *Stenotrophomonas maltophilia*
  - *Achromobacter* spp.
- Non-“Big 4” Gram negatives
Mucoid P. aeruginosa

Inquilinus
Is there a better way?

• Mucoid *P. aeruginosa*
  – “Easily identifiable”
  – Oxidase + PLUS mucoid = *P. aeruginosa*

• Recent study:
  – Examined 118 mucoid, oxidase + isolates by:
    • Brief biochemical testing
    • PCR identification with *gyrB*
Identification methods

• Biochemical testing
  – Arginine hydrolysis
  – Growth at 42°C
  – β-hemolysis

• Definitive identification “gold standard”
  – gyrB rrtPCR
    gyr PA-398: (22nt) 5’-CCT GAC CAT CCG TGC CCA C-3’
    gyr PA-620: (20nt) 5’-CGC AGC AGG ATG CCG AGC CC-3’
Results

<table>
<thead>
<tr>
<th>Assay Combination</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value (PPV)</th>
<th>Negative Predictive Value (NPV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucoid Positive Oxidase Positive</td>
<td>100.0%</td>
<td>16.7%</td>
<td>95.7%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Mucoid Positive Oxidase Positive Arginine Positive</td>
<td>94.6%</td>
<td>83.3%</td>
<td>99.1%</td>
<td>45.5%</td>
</tr>
<tr>
<td>Oxidase Positive Mucoid Positive β-Hemolysis Positive</td>
<td>79.5%</td>
<td>83.3%</td>
<td>98.9%</td>
<td>17.9%</td>
</tr>
<tr>
<td>Oxidase Positive Mucoid Positive 42°C Growth Positive</td>
<td>91.1%</td>
<td>16.7%</td>
<td>87.2%</td>
<td>9.1%</td>
</tr>
</tbody>
</table>
Conclusions

• Continue to do only oxidase testing on mucoid isolates
  – If positive, call *P. aeruginosa*
  – If negative, use molecular techniques to identify
Big 4 testing

- Identification of other GNRs typically seen in CF (Big 4)
  - Non-mucoid *P. aeruginosa*
  - *Burkholderia cepacia* complex species
  - *Stenotrophomonas maltophilia*
  - *Achromobacter xylosoxidans*
Big 4 testing

- Often difficult to identify, due to CF adaptation
- *S. maltophilia* is often easier because it is oxidase –
- Developed multiplex PCR for ID
  - Skip biochemical testing, go straight to PCR
  - If PCR negative, non-Big 4
Oxidase + nonmucoid → Mucoid, oxidase + → ID: mucoid

P aeruginosa

PCR
Non-Big 4 testing

• How do we ID non-Big 4 GNRs?
• Sequence:
  – Previously V3
  – Now evaluating PCR identification
Example of V3 sequencing

V3 F’
CGGYCCAGACTCCTACCGGG

=====CCAGACTCCTACGGGAGGCAGCAGTGGG
GGAATTGTGGACAATGGGGGAAACCCTGATC
CAGCCATCCCGCGTGTGCGATGAAGGCCTT
CGGGTTGTAAAGCAGACTTTTGGCAGGAAAGAA
ACGTCGTGGGGTTAATACCCCGCGAAACTGAG
CGGTACCTGCAGAATAAGCACCAGGCTAAGTA
CGTGCCAGCAGCCCGCGGGTAAAA

GTGCCAGCAGCCCGCGGGTAAA V3 R’
How good is sequence data?

Get identical matches with *Achromobacter xylosoxidans*, *Achromobacter denitrificans*, and *Achromobacter insolitus*.
## How good is sequence data?

<table>
<thead>
<tr>
<th>Ref strain</th>
<th>Delta Top score (% pairwise comparison)</th>
<th>V3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMG 6003</td>
<td>0 (100%)</td>
<td>A. insolitus</td>
</tr>
<tr>
<td>A. insolitus</td>
<td></td>
<td>A. xylosoxidans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. dentrificans</td>
</tr>
<tr>
<td>FJ810080</td>
<td>0 (100%)</td>
<td>A. xylosoxidans</td>
</tr>
<tr>
<td>A. dentrificans</td>
<td></td>
<td>A. dentrificans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. insolitus</td>
</tr>
<tr>
<td>EU877076</td>
<td>0 (100%)</td>
<td>A. xylosoxidans</td>
</tr>
<tr>
<td>A. xylosoxidans</td>
<td></td>
<td>A. dentrificans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. insolitus</td>
</tr>
<tr>
<td>ATCC 9220</td>
<td>0 (100%)</td>
<td>A. xylosoxidans</td>
</tr>
<tr>
<td>A. xylosoxidans</td>
<td></td>
<td>A. dentrificans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. insolitus</td>
</tr>
</tbody>
</table>
How good is sequence data?

<table>
<thead>
<tr>
<th>Ref strain</th>
<th>Delta Top score (% pairwise comparison)</th>
<th>~500 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMG 6003</td>
<td>0 (100%)</td>
<td>A. insolitus</td>
</tr>
<tr>
<td>A. insolitus</td>
<td></td>
<td>A. xylosoxidans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. dentrificans</td>
</tr>
<tr>
<td>FJ810080</td>
<td>0 (100%), S=961</td>
<td>A. dentrificans</td>
</tr>
<tr>
<td>A. dentrificans</td>
<td>4 (100%), S=957</td>
<td>A. xylosoxidans</td>
</tr>
<tr>
<td></td>
<td>11 (99.6%) S=950</td>
<td>A. insolitus</td>
</tr>
<tr>
<td>EU877076</td>
<td>0 (100%), S=917</td>
<td>A. xylosoxidans</td>
</tr>
<tr>
<td>A. xylosoxidans</td>
<td>119 (96.3%), S=798</td>
<td>MCRT A dent.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCRT A insol.</td>
</tr>
<tr>
<td>ATCC 9220</td>
<td>0 (100%), S=961</td>
<td>A. xylosoxidans</td>
</tr>
<tr>
<td>A. xylosoxidans</td>
<td>6, (99.8%), S=955</td>
<td>A. dentrificans</td>
</tr>
<tr>
<td></td>
<td>6, (99.8%), S=955</td>
<td>A. insolitus</td>
</tr>
</tbody>
</table>
How good is sequence data?

<table>
<thead>
<tr>
<th>Ref strain</th>
<th>Delta Top score (% pairwise comparison)</th>
<th>~1500bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMG 6003 A. insolitus</td>
<td>0, (100%), S=2713 18 (99.7%), S=2695 46 (99.5%), S=2667</td>
<td>A. insolitus A. dentrificans A. xylosoxidans</td>
</tr>
<tr>
<td>FJ810080 A. dentrificans</td>
<td>0, (100%), S=2813 63, (99.8%), S=2750 71, (99.8%), S=2741</td>
<td>A. dentrificans A. xylosoxidans A. insolitus</td>
</tr>
<tr>
<td>EU877076 A. xylosoxidans</td>
<td>0, (100%), S=2667 160, (98.3%), S=2507 166, (98.25%), S=2501</td>
<td>A. xylosoxidans MCRT A. dentrificans MCRT A. insolitus</td>
</tr>
<tr>
<td>ATCC 9220 A. xylosoxidans</td>
<td>0, (100%), S=2752 5, (99.9%), S=2747 11, (99.9%), S=2741</td>
<td>A. xylosoxidans A. insolitus A. dentrificans</td>
</tr>
</tbody>
</table>
How good is sequence data?

- *A. xylosoxidans*, *A. dentrificans* (*A. xylosoxidans* subsp. *dentrificans*), and *A. insolitus* are indistinguishable at V3 (200bp), P1/P3 (500bp) and 1500 bp regions.

- Therefore, isolates that key out as any of those organisms must be identified as *Achromobacter* spp.

- Is there a significant clinical difference between *A. xylosoxidans* and *Achromobacter* spp.? – i.e. does it matter?
Respiratory viruses
Viral detection in CF

- Recently completed a prospective study of viral infections in CF
  - Enrolled 44 subjects
  - Followed through 2 viral seasons
  - Study visits at quarterly clinic visits, sick visits and hospitalizations
  - Evaluated: NP samples (wash or NP swab), OP swabs, expectorated sputum
Viral detection in CF

- Subject age: 12.8 (sd 3.0)
- BMI: 45.9 (27.1)
- FEV$_1$ % predicted: 94.2 (18.2)
- Total study visits: 390
- Samples analyzed by PCR: 460
Viral detection in CF

- PCR detection of RSV, hMPV, influenza, parainfluenza, adenovirus, coronavirus, rhinovirus
  - RT-PCR assays for total 11 viruses
  - Appropriate controls for extraction, inhibition
- Subjects with no positive samples: 9.1%
- Subjects with 1 positive sample: 27.3%
- Subjects with 2 or more positive samples: 63.6%
- Total of 40 positive subjects (90.0%) at some point during study
### Viral detection in CF

<table>
<thead>
<tr>
<th>Virus</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV</td>
<td>2</td>
<td>4.5</td>
</tr>
<tr>
<td>MPV</td>
<td>6</td>
<td>13.6</td>
</tr>
<tr>
<td>Influenza</td>
<td>6</td>
<td>13.6</td>
</tr>
<tr>
<td>Paraflu</td>
<td>11</td>
<td>27.5</td>
</tr>
<tr>
<td>Adeno</td>
<td>5</td>
<td>11.4</td>
</tr>
<tr>
<td>Corona</td>
<td>5</td>
<td>11.4</td>
</tr>
<tr>
<td>Rhino</td>
<td>36</td>
<td>81.8</td>
</tr>
</tbody>
</table>
Summary

• Concordance of upper and lower airway results
  – Rhinovirus:
    • 79 concordant pairs: 14 +/-, 65 -/-
    • 13 discordant pairs: equally divided between upper and lower airway positives (p=0.78)
  – Similar results for combined other viruses (p=0.71)
  – Trend toward better detection from sputum:
    • Influenza A, adenovirus, coronavirus
Non-culturable organisms in CF
Non-culturable organisms in CF

• Several recent studies:
  – Worlitzsch, 2002: anaerobic areas within CF lung
  – Rogers, 2004: used T-RFLP profiling to evaluate 71 samples from 34 CF patients
    • Most commonly found *P. aeruginosa* (88%)
    • Also *Porphyromonas* and *Prevotella* (71%), *Craurococcus roseus* (62%), *Ralstonia* (35%)
    • *Selenomonas, Streptococcus intermedius* (32%)
Non-culturable organisms in CF

• 2nd study in 2009:
  – 12 patients: 8 CF, 4 COPD
  – Plated samples and amplified nucleic acids (culture dependent)
    • 8.8 (5.8) TRF band lengths per profile
  – Amplified nucleic acids directly from sputum (culture independent)
    • 16.3 (5.8) TRF band lengths per profile
  – Suggests organisms are being lost on cultures
Non-culturable organisms in CF

• What does this mean?
  – Are they normal flora?
  – Are they colonizers?
  – Are they agents of co-infection?
  – Are they pathogenic?

• Need clinical correlates
Non-culturable organisms in CF

- Clinical relevance of *Streptococcus milleri* group (SMG) addressed by Sibley (2008)
- Used T-RFLP to examine individual patients with linked clinical data
  - Index patient followed for 1 year
    - Found SMG by T-RFLP (and culture)
    - 3 pulmonary exacerbations: each responded to anti-SMG antibiotics
  - Examined 18 other pulmonary exacerbations over 6 months
    - 7/18 (39%) associated with SMG
Summary

- Molecular diagnostics are currently available for bacterial identification.
- Molecular diagnostics can be used for the detection of viral infections from available samples in CF.
- Molecular diagnostics have the potential to unlock the complexity of CF airway infections.
Contributors

• Seattle Children’s Center for CF Microbiology
  – Anne Marie Buccat, MS
  – Xuan Qin, PhD

• Viral study
  – Janet Englund, MD
  – Jane Kuypers, PhD
  – Julia Emerson, MD, MPH