The –Omics Revolution Meets Microbiology and Infectious Diseases

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DISCLOSURE

• Receives Royalties from Biomerieux Inc. for DiversiLab
• Past Research Support from Roche Diagnostics
Texas Children’s Hospital
Molecular Microbiology Team
From Microbial Genomics to Diagnostic Microbiology: BENCH TO BENCH

- POSITIVE CULTURES

- Pyrosequencing

- Gram Stain

- Real-Time PCR

- Rep-PCR
Highlights: A Summary

• Pathogen Identification
• Drug-Resistant Pathogens
  – Strain Tracking
• Enteric Pathogens
  – Gastroenteritis
  – Diagnosis of Clostridium difficile Infections
• Respiratory Pathogens
  – Challenging the single virus and single bacterial species paradigm
Difficult-to-Identify Pathogens

- Organisms requiring DNA sequencing for identification at Texas Children’s Hospital
- Cystic fibrosis
  - *Burkholderia cepacia* complex
  - Atypical *Pseudomonas aeruginosa*
- *Microbacterium* spp.
  - Considered as blood culture contaminant
- *Rothia mucilaginosa*
  - Neutropenic children with hematologic malignancies

Approximately 20-30 Vitek workups per day in the TCH Microbiology Laboratory.

Between December 2003 and July 2006, a total of 414 cultured isolates (312 children) were submitted and processed for DNA pyrosequencing.

Approximately 90% (n=372) of isolates were identified and reported by DNA pyrosequencing.

78 different genera: 51% to species level

Rothia mucilaginosa
Clinical Case

• 20 year-old male with relapsed chronic myelogenous leukemia (CML)
• Status post-bone marrow transplantation (x3) and failed engraftment
• Developed meningitis and encephalopathy unresponsive to antibiotics
• Severe diffuse cerebral edema
• Diffuse ependymitis
Direct Diagnosis of *Rothia mucilaginoso* Infection

- Direct PCR amplification from cerebral meninges at autopsy
- DNA Pyrosequencing identified *Rothia mucilaginoso*
Identification of *Pseudomonas aeruginosa* in CF Cultures

- Sputum samples collected from patients with cystic fibrosis at each quarterly visit.
- *P. aeruginosa* identified through a combination of culture on selective media and biochemical testing.
Identification of *Pseudomonas aeruginosa* by Pyrosequencing

- Some *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis are difficult to identify by conventional microbiology methods due to their phenotypic diversity.
- Many *Pseudomonas* isolates from respiratory cultures of patients were submitted for pyrosequencing bacterial identification.
Pyrosequencing Identification of *Pseudomonas aeruginosa*

Bacterial DNA Extraction

Amplification of V1 and V3 variable regions of 16S rDNA

Pyrosequencing

Sequence analysis and final identification

- S000006524 1.000 1388 Pseudomonas aeruginosa, ATCC 27853, AF094719
- S000007259 1.000 1432 Pseudomonas aeruginosa, AF225956
- S000007359 1.000 1416 Pseudomonas aeruginosa, M34133
- S000007839 1.000 1445 Pseudomonas aeruginosa, #985, AB037556
Pyrosequencing Identification of *Pseudomonas aeruginosa*

- For more than 3 years, 47 isolates from sputum and BAL samples were identified as *Pseudomonas aeruginosa*.
Cellulomonas denverensis (Culture Acc XXXXX) was identified by DNA pyrosequencing.

This test was developed and its performance characteristics determined by Texas Children's Hospital. It has not been cleared or approved by the U.S. FDA.
Multi-Drug Resistant

*Pseudomonas aeruginosa*

- Resistant to all antibiotics in 2 or more groups below
  - Resistant to all aminoglycosides tested
    - Tobramycin
    - Gentamicin
    - Amikacin
  - Resistant to all quinolones tested
    - Ciprofloxacin
  - Resistant to all beta lactams tested
    - Ceftazidime
    - Meropenem
    - Timentin
    - Piperacillin
    - Ticarcillin
    - Aztreonam
Molecular Typing of *Pseudomonas aeruginosa* in Cystic Fibrosis

- Several studies utilizing molecular typing of *Pseudomonas aeruginosa* in cystic fibrosis in late 1990s and 2000s.
- Methodology included mostly PFGE and RAPD, no study used rep-PCR.
- No study has specifically focused on MRPA.
- However, in 2 studies, the dominant clone was found to be MRPA.
Which bacterial clone or strain is responsible for clusters of hospital-associated infections?

One or many clones?
Molecular Typing at TCH

CF Respiratory Samples → MRPA Culture → Infection Control Requests Hold for Typing

Bacterial DNA Extraction → rep-PCR

Automated Fingerprinting → Results (Dendrogram)
Multi-Drug Resistant
*P. aeruginosa* Clusters by Year

Collaboration with Peter Hiatt, M.D. and Jeffrey Starke, M.D. at Texas Children’s Hospital, Houston, TX
DiversiLab Report

Newly submitted *Enterobacter cloacae* isolates XXXX and XXXXXX are indistinguishable by rep-PCR and may represent the same clone. These data suggest a genetic relationship may exist among these isolates. A common point source for these bacterial isolates may exist. Newly submitted isolate XXXXX is similar to isolates XXXX and XXXXXX.

Newly submitted isolates XXXXXX is clearly distinguishable from all other isolates in this sample set by rep-PCR. Based on these data, there is no evidence of a genetic relationship involving this isolate. Stated differently, the data do not suggest horizontal transmission or a common point source for this bacterial isolate.

When compared to the Ukent *Enterobacter cloacae* Library, newly submitted isolates XXXXXX, XXXX, and XXXXXX are similar to previously submitted isolate XXXXXXX. Newly submitted isolate XXXXXX is clearly distinguishable from all isolates in the library.
Molecular Typing Redefined
Infection Control in CF Center

• Dominant MRPA clone was focus of infection control efforts
  – Facile transmission between patients
• Children were tracked, treated promptly, and placed in contact isolation at TCH
• Parents were counseled; multiple interventions
• As of July 2008, only 2 new patients were identified with the dominant clone in approximately 18 months.
  – MRPA is under control locally
Challenges in Diagnosing Pediatric Infections of the Digestive System

• Variety of Etiologies to Consider
  – Bacterial, Viral, Parasitic
  – New, Re-Emerging, or Under-Appreciated Agents

• Colonization versus Infection
  – Human Microbiota and Microbiome
  – Bacteria and Viruses
  – Example of *C. difficile* Colonization in Infants

• Limitations of Current Stool-Based Strategies
  – Bacteriologic Culture
  – Antigen Detection
Clostridium difficile

Culture on CCFA Agar

Gram stain

Originally labeled “Bacillus difficilis” in 1930s – the “difficult one”
Summary of *C. difficile* PCR Validation

- **22 true positive samples** based on stool anaerobic culture / PCR / immunoassay
  - 19 positive samples or **86% sensitivity** for direct real-time PCR
  - 9 positive samples or **37.5% sensitivity** for direct toxin testing
    - Low sensitivity may reveal limitations with pediatric samples
- **122 true negative samples** based on culture/ PCR / Immunoassay
  - 117 negative samples or **96% specificity** by direct real-time PCR
  - 122 negative samples or **100% specificity** by direct toxin testing
- Association for Molecular Pathology meeting in Orlando (November 2006)
  - S. M. Paule *et al.* *J Mol Diagnostics* 2006;8:653. (Northwestern)
  - Real-time PCR (*tcdB* only)
    - 77% sensitivity and 99% specificity when compared to toxigenic culture
    - Excellent correlation between toxin gene detection and toxigenic culture
    - “… real-time PCR provides the best combination of speed and accuracy.”
CDF Yearly Volumes & Positivity Rates

Average EIA Positivity Rate
8.0%

Average PCR Positivity Rate
18.1%
C. difficile PCR Report

C DIFFICILE TOX PCR

Negative for toxigenic Clostridium difficile.

Note: Published data indicate that up to 65% of infants may have asymptomatic colonization of toxigenic C. difficile due to the immature nature of the digestive tract in infants up to 1 year of age. Other causes of diarrhea, particularly enteric viruses, should be considered in this age group.

Methodology: Bacterial DNA, if present, was extracted from a stool specimen. Real time PCR with primers and probes specific for Clostridium difficile toxin producing genes tcdA and tcdB was performed. This test was developed and its performance characteristics determined by Texas Children's Hospital. It has not been cleared or approved by the U.S. FDA.
Challenge: Diagnosis of Respiratory Tract Infections

- Continually expanding repertoire of respiratory viruses
- Complexity of bacterial colonization in ventilator-associated infections
BACTERIAL/FUNGAL IDENTIFICATION

DNA SEQUENCING

Today

PYROSEQUENCING

Today

REAL-TIME PCR

Today

ARRAYS AND MICROARRAYS

Have arrived
Liquid Bead Arrays

Antibodies

Proteins

Peptides

Oligonucleotides

Luminex-100

Panomics Inc.
Respiratory viral panel (Luminex) was FDA-approved on Jan. 3, 2008
Array-Based Respiratory Virus Detection

- Liquid bead arrays by Respiratory Multi-code-PLx Assay (RMA) and microsphere flow cytometry (Luminex)
- RMA detected respiratory viruses in 71.8% versus 23.3% of clinical specimens by DFA and viral culture
- Nasal wash samples from 5 year-old children

### RVP Report Text from ViraCor -1

**RESP VIRAL PANEL**

NASAL WASH Results as Follows:

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metapneumovirus</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>POSITIVE</td>
</tr>
<tr>
<td>Influenza A</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Influenza A subtype H1</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Influenza A subtype H3</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Influenza B</td>
<td>Not Detected</td>
</tr>
<tr>
<td>RSV A</td>
<td>Not Detected</td>
</tr>
<tr>
<td>RSV B</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Parainfluenza 1</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Parainfluenza 2</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Parainfluenza 3</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Not Detected</td>
</tr>
</tbody>
</table>

REFERENCE VALUE FOR ALL ANALYTES: Not Detected.

**Viracor**
Respiratory Virus Panel Report (cont.)

For in vitro diagnostic use. Respiratory Viral Panel is a product of Luminex Corporation performed by XXXX, a CLIA certified laboratory.

The performance characteristics for this specimen are unknown. This specimen type has not been cleared by the FDA. Results should be used in conjunction with clinical findings.

For influenza A specimens reported as "Not Detected" for both the matrix gene target and the hemagglutinin gene target, the FDA cleared RVP package insert states the following: "It is recommended that specimens found to be negative for influenza A matrix gene target and influenza A hemagglutinin gene target in a respiratory viral panel nucleic acid detection assay be confirmed by cell culture. Negative results do not preclude respiratory virus infection and should not be used as the sole basis for diagnosis, treatment, or other management decisions."

TEST PERFORMED BY
XXXXX
Viracor
DNA Microarrays for Pathogen Detection in a Microbial World
The ViroChip

- Microarray-based viral detection
  - 70mer spotted arrays
- ViroChip microarray recognizes greater than 140 viruses
- Human rhinovirus detected post-infection (panel A)
  - Nasal lavage
- Human rhinovirus detection in natural colds (panel B)
  - Nasal lavage
- Human parainfluenza virus 1 detected (panel C)
  - Nasal lavage

PhyloChip – high-density oligonucleotide microarray for bacterial detection

More than 8,000 taxa / chip
- At least 11 probes per taxon

16S rRNA gene sequencing detected reduction from 16.2 to 5.6 (mean number of) bacterial species with antibiotic therapy
• Bacterial PhyloChip studies in human endotracheal aspirates
  • intubated ICU patients
  • Sampling at beginning of parenteral antibiotic versus 4-10 days of therapy
• Loss of bacterial diversity was correlated with ventilator-associated pneumonia during antibiotic therapy

From Microbial Genomics to Diagnostic Microbiology: BENCH TO BENCH

- POSITIVE CULTURES

Pyrosequencing → Gram Stain → Real-Time PCR

Rep-PCR
YOUR BODY IS A PLANET

Of the 100 trillion cells inside each one of us, only 10 percent are actually human. The rest belong to aliens: bacteria, fungi, and other microbes.

BY JOSIE FLANAGAN

We may not realize it, but each one of us is a walking ecosystem. Minuscule, sightless, ribbed worms called nematodes live in the floor of the eyes, toasting easter eggs of skin cells. Microscopic worms live on the tongue, lips, and skin and in the intestines. Damp regions like nipples and pubic hair may harbor five grams inside. Certain cells, perhaps of all are the self-replicating, circular pieces of DNA that infected ancient humans and still make up about 9 percent of our genome. Most of the time we share our bodies harmoniously with the 90 trillion or so microbes. But sometimes these microbes turn on themselves, when blood-supplying tissue becomes clogged, as when blood-supplying tissue becomes clogged, or when herpes simplex or human papillomavirus causes lesions on the tongue and lips. Others spread and their relatives can spread from the mouth and throat and into other areas of the skin, including the scalp and cornea.

1. **NEMATODES**
   - The nematode worm, or pinworm, is a common parasite that infects both humans and animals. Nematodes live in the floor of the eyes, toasting easter eggs of skin cells. Microscopic worms live on the tongue, lips, and skin and in the intestines. Damp regions like nipples and pubic hair may harbor five grams inside.

2. **BACTERIA AND Fungi**
   - Bacteria are everywhere, living in and on every surface of the body, including the scalp and cornea. Some bacteria are beneficial, while others can cause disease.

3. **HERPES SIMPLEX**
   - Herpes simplex is a virus that can cause lesions on the tongue and lips. It is transmitted through direct contact with infected skin.

4. **HUMAN PAPILLOMAVIRUS**
   - Human papillomavirus (HPV) can infect humans, causing a variety of lesions and warts. At least 30 strains of HPV are sexually transmitted, and the CDC estimates that over 20 million Americans have genital warts at some point. Of the 15 confirmed strains, HPV types 11, 16, and 18 are the most common.

5. **HEAD LOSE**
   - Pseudocalculus formation occurs when the hair follicles become clogged by the sebum of the skin. This can lead to the formation of a hard lump, or calculus, on the scalp.

6. **DENTAL DOTOPODOSIS**
   - If you don’t brush your teeth regularly, the bacteria in your mouth can build up and form plaque, which can lead to tooth decay and gum disease.

7. **FOCAL VERSUS**
   - Focal versus is a disease that affects the skin, causing it to thicken and become scaly. It is often caused by a virus, such as the chickenpox virus.

8. **STROPHOCODOSIS**
   - Strophocodosis is a condition that affects the skin, causing it to become red and inflamed. It is often caused by a virus, such as the chickenpox virus.

(Glausiusz J. Discover June 2007)
The Human Microbiome Project: Indigenous Microbiota and Microbiome

>60% novel bacteria
>80% nonculturable bacteria


Firmicutes include *Lactobacillus* spp.


Mixed Microbial Communities

Human Colon
800-1000 species
*Firmicutes*
*Bacteroidetes*
Is it Microbial Composition or Functional Genomics?

A core gut microbiome in obese and lean twins

Peter J. Turnbaugh¹, Micah Hamady³, Tanya Yatsunenko¹, Brandi L. Cantarel³, Alexis Duncan², Ruth E. Ley¹, Mitchell L. Sogin⁶, William J. Jones⁷, Bruce A. Roe⁸, Jason P. Affourtit⁹, Michael Egholm⁹, Bernard Henrissat⁵, Andrew C. Heath², Rob Knight⁴ & Jeffrey I. Gordon¹

Highlights: A Summary

• Bacterial and Fungal Pathogen Identification
• Drug-Resistant Pathogens
  – Strain Tracking
• Enteric Pathogens
  – Gastroenteritis
  – Diagnosis of *Clostridium difficile* Infections
• Respiratory Tract Infections, Viruses and Bacterial Communities
  – Respiratory Virus Panels and ViroChip
  – Challenging the single species paradigm
The End