BCM
Baylor College of Medicine

Texas Children's Hospital

The –Omics Revolution Meets Microbiology and Infectious Diseases

James Versalovic, M.D., Ph.D.
Director, Division of Molecular Pathology
Director, Microbiology Laboratories
Department of Pathology
Texas Children's Hospital

Associate Professor of Pathology, Pediatrics, Molecular Virology & Microbiology, Molecular and Human Genetics
Baylor College of Medicine

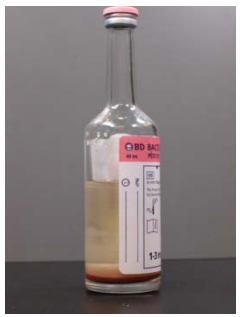
DISCLOSURE

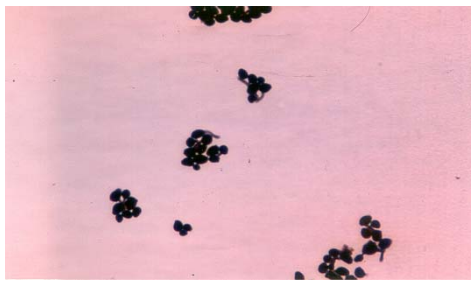
- Receives Royalties from Biomerieux Inc. for DiversiLab
- Past Research Support from Roche Diagnostics

Texas Children's Hospital Molecular Microbiology Team

From Microbial Genomics to Diagnostic Microbiology : BENCH TO BENCH

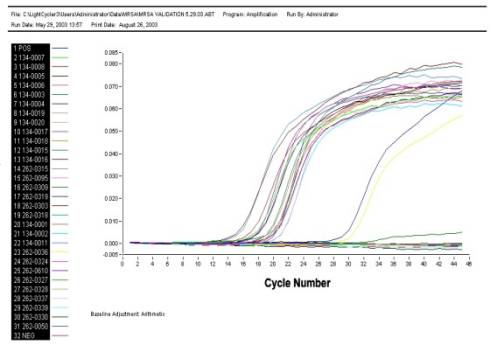
- **POSITIVE CULTURES**

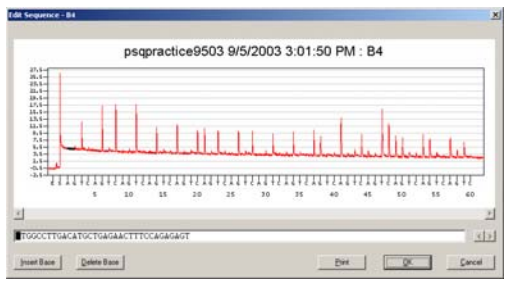




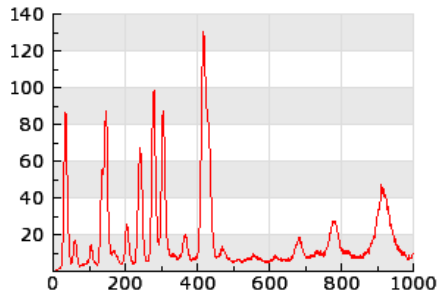
Gram Stain

Real-Time PCR





Pyrosequencing



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

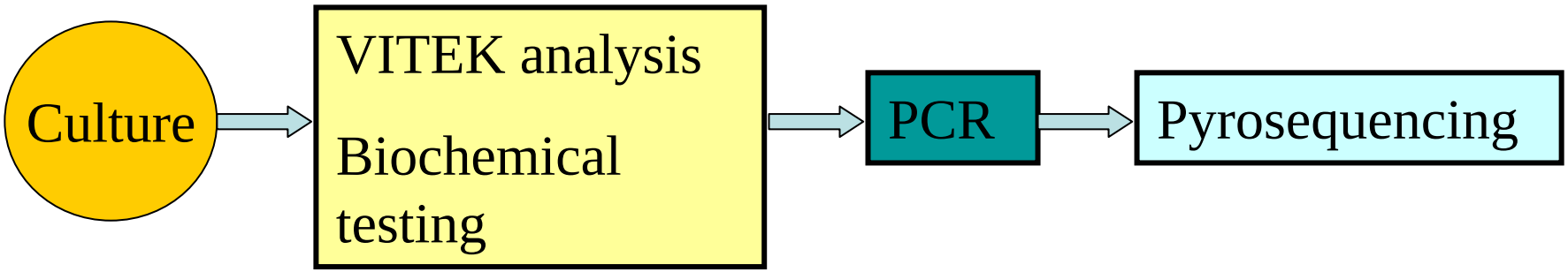
Difficult-to-Identify Pathogens

- 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

Luna RA *et al*. (2007) *J Clin Microbiol* 45:2985-2992.

Biochemical – Molecular Identification

Molecular Microbiology Work Flow



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.

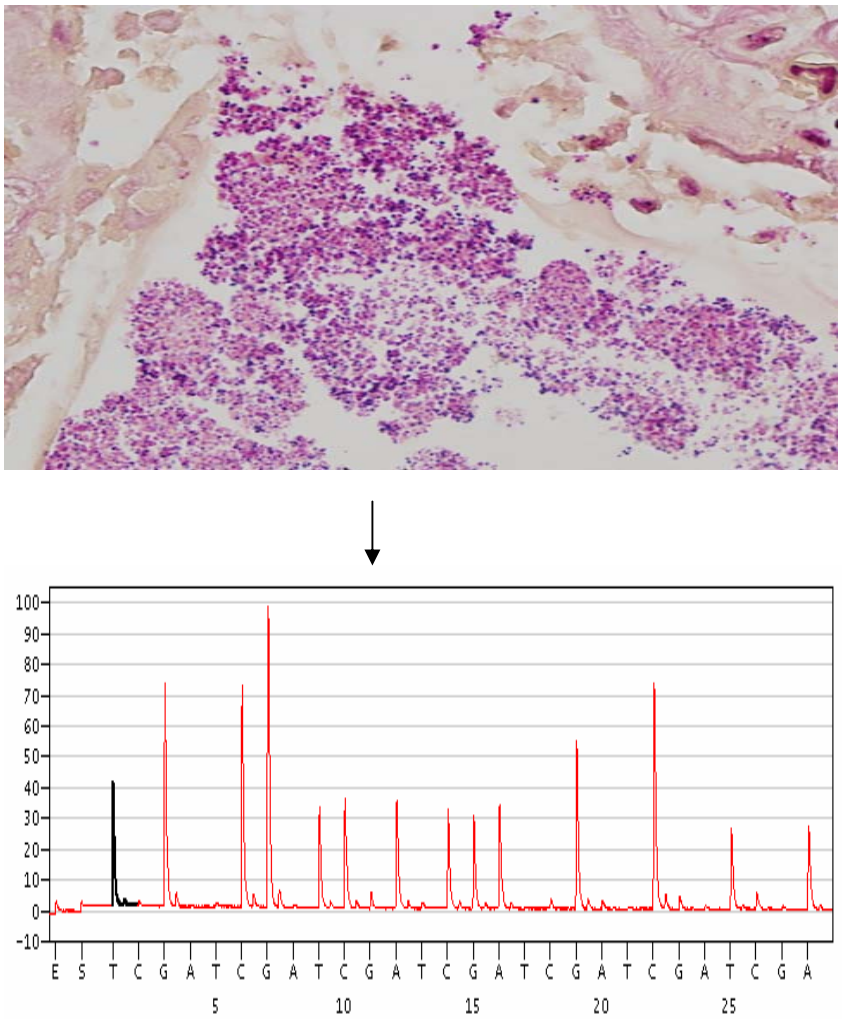
78 different genera : 51% to species level

Luna RA *et al*. (2007) *J Clin Microbiol* 45:2985-2992.

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

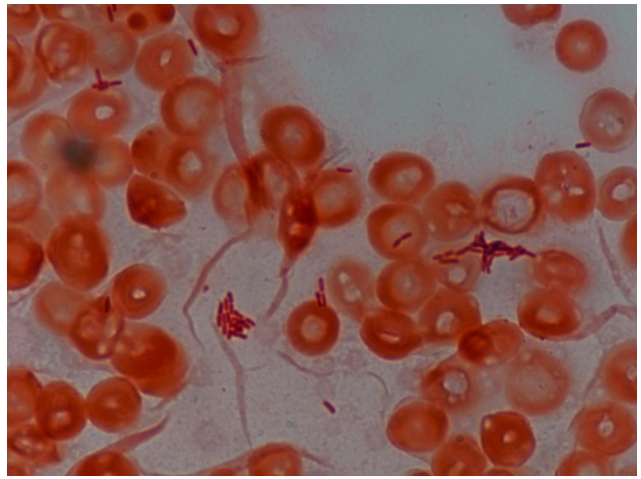
Direct Diagnosis of *Rothia mucilaginosa* Infection

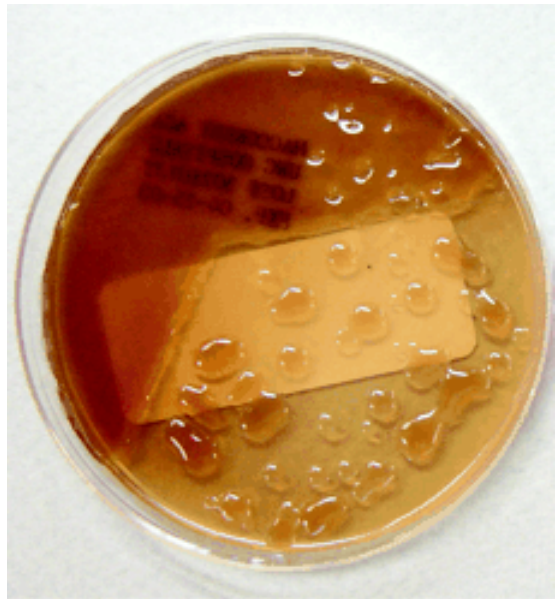


- Direct PCR amplification from cerebral meninges at autopsy
- DNA Pyrosequencing identified *Rothia mucilaginosa*

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

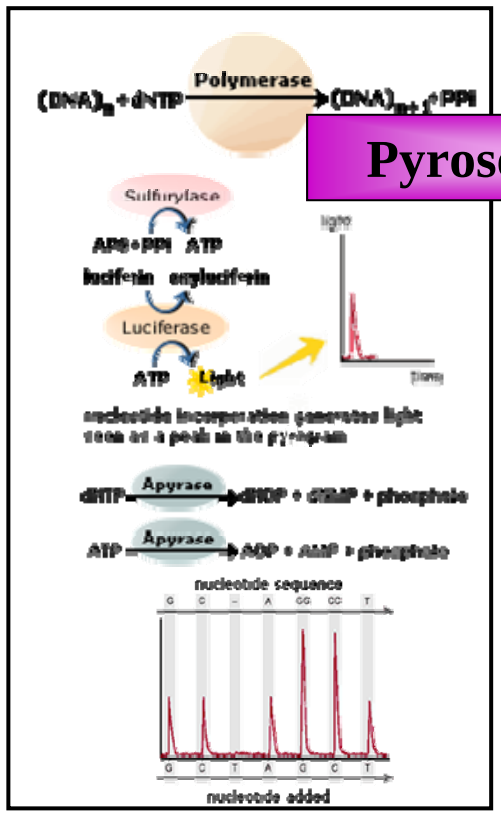
- Some *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis are difficult to identify by conventional microbiology methods due to their phenotypic diversity.
- Many *Pseudomonas* isolates from respiratory cultures of patients were submitted for pyrosequencing bacterial identification.

Pyrosequencing Identification of *Pseudomonas aeruginosa*

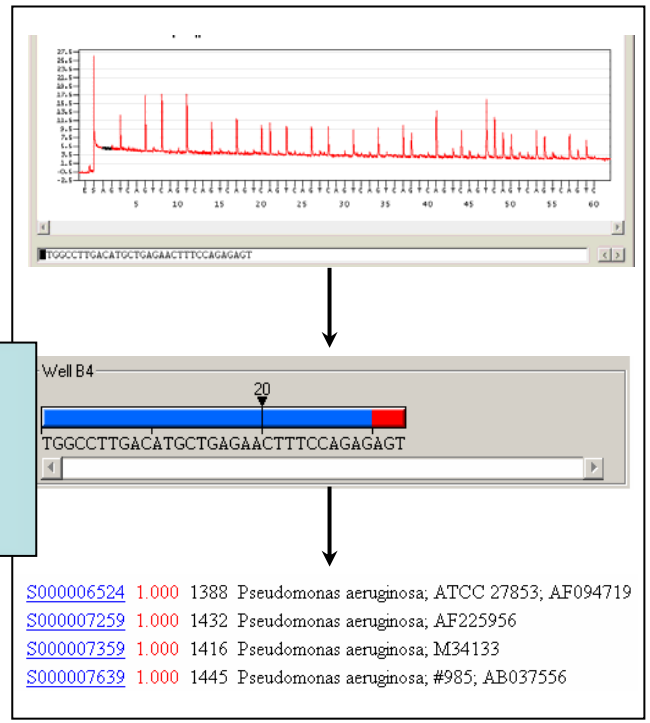
Bacterial DNA Extraction

Amplification of V1 and V3 variable regions of 16S rDNA

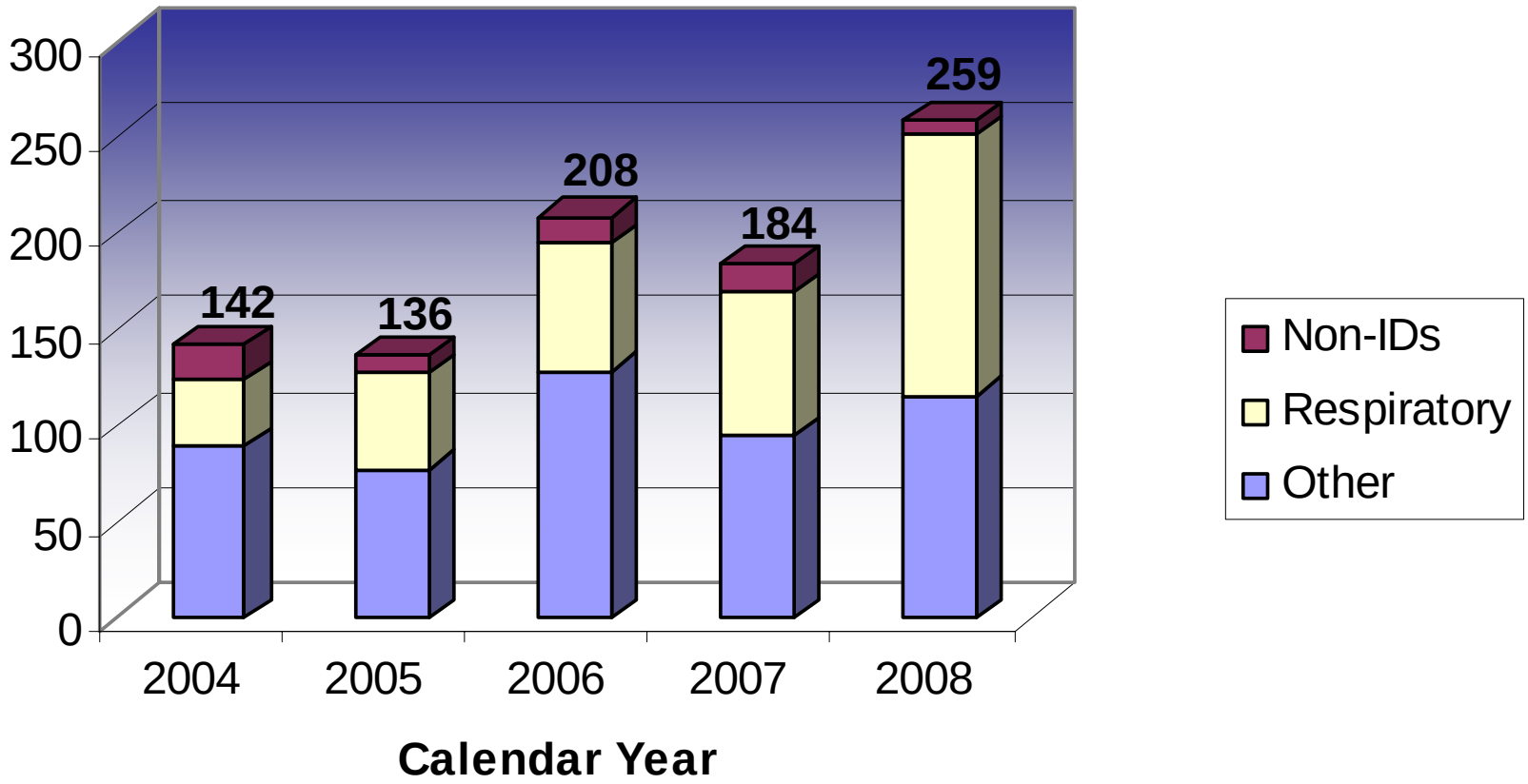
Pyrosequencing



Sequence analysis and final identification

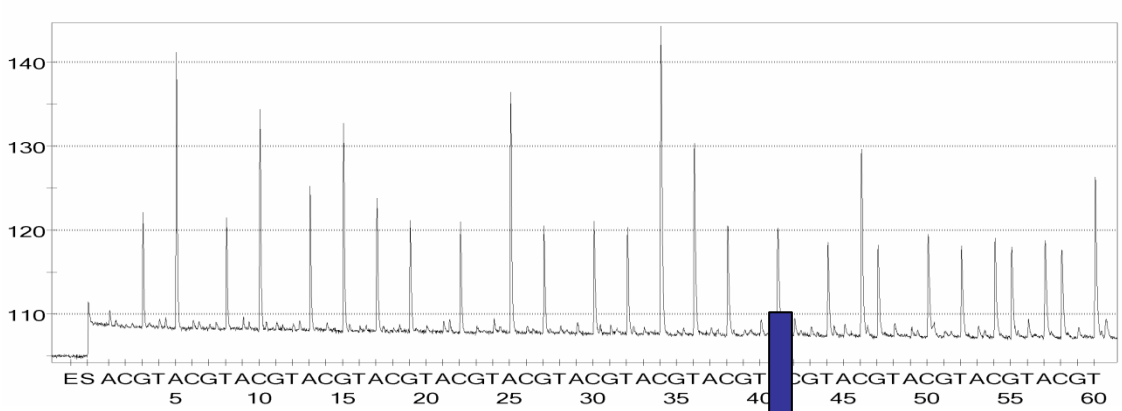


Bacterial PSQ ID Volumes



Pyrosequencing Identification of *Pseudomonas aeruginosa*

- For more than 3 years, 47 isolates from sputum and BAL samples were identified as *Pseudomonas aeruginosa*.



S000000348 - not_calculated 1.000 1427 Pseudomonas aeruginosa; H814; AB037563
S000000739 - not_calculated 1.000 1431 Pseudomonas aeruginosa; #885; AB037555
S000000939 - not_calculated 1.000 1441 Pseudomonas aeruginosa; #796; AB037553
S000002283 - not_calculated 1.000 1297 Pseudomonas aeruginosa; AF137358
S000002377 - not_calculated 1.000 1459 Pseudomonas aeruginosa; AL98; AJ249451

DNA Pyrosequencing Report

PYROSEQUENCING ID

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*

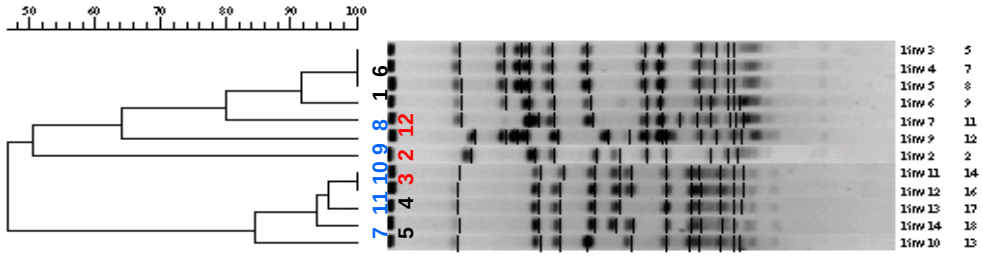
- Resistant to all antibiotics in 2 or more groups below
  - Resistant to all aminoglycosides tested
    - Tobramycin
    - Gentamicin
    - Amikacin
  - Resistant to all quinolones tested
    - Ciprofloxacin
  - Resistant to all beta lactams tested
    - Ceftazidime
    - Meropenem
    - Timentin
    - Piperacillin
    - Ticarcillin
    - Aztreonam

Molecular Typing of *Pseudomonas aeruginosa* in Cystic Fibrosis

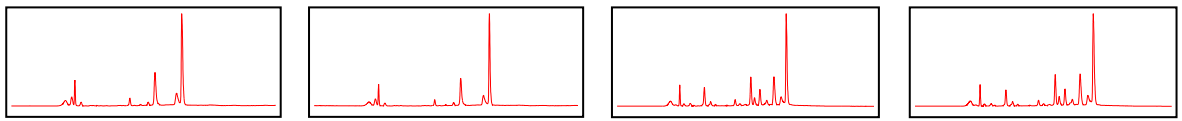
- Several studies utilizing molecular typing of *Pseudomonas aeruginosa* in cystic fibrosis in late 1990s and 2000s.
- Methodology included mostly PFGE and RAPD, no study used rep-PCR.
- No study has specifically focused on MRPA.
- However, in 2 studies, the dominant clone was found to be MRPA.
  - Jones AM, et al. Lancet. 2001 Aug 18;358(9281):557-8.
  - Armstrong D, et al. J Clin Microbiol. 2003 May;41(5):2266-7.

MOLECULAR EPIDEMIOLOGY – DNA FINGERPRINTING

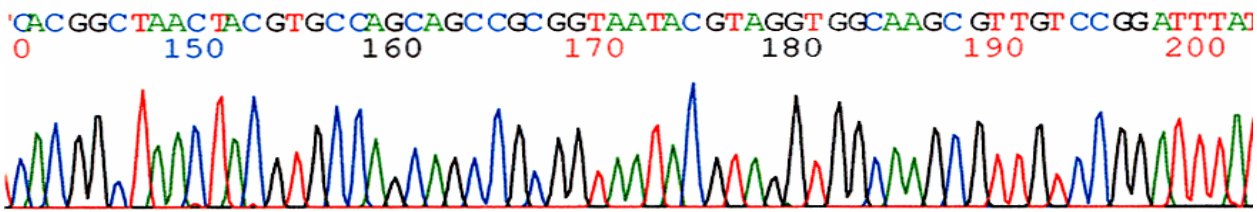
PULSED-FIELD GEL ELECTROPHORESIS



REP-PCR



DNA SEQUENCING



Which bacterial clone or strain is responsible for clusters of hospital-associated infections?

One or many clones?

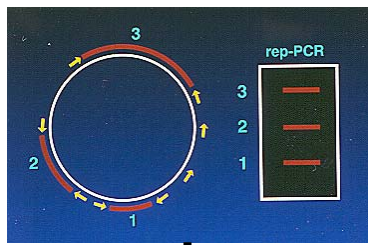
Molecular Typing at TCH

CF Respiratory Samples

MRPA Culture

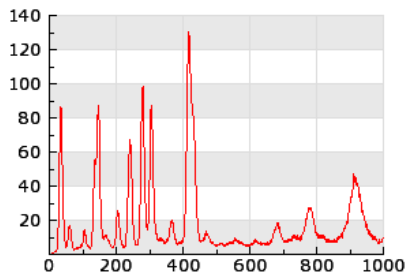
Infection Control Requests Hold for Typing

Bacterial DNA Extraction

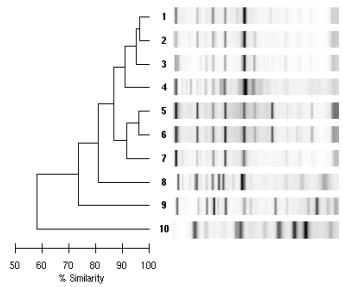


rep-PCR

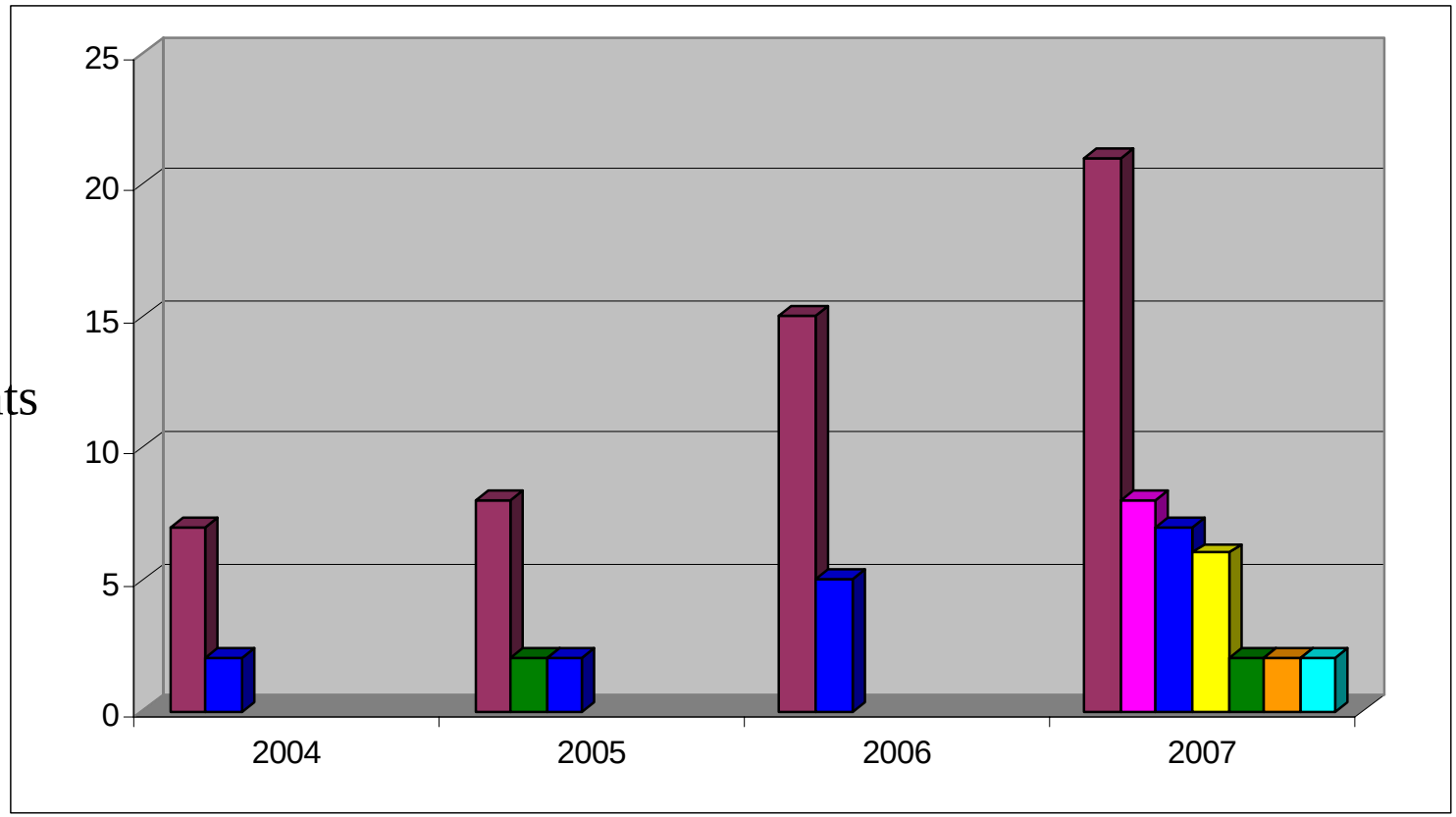
Automated Fingerprinting



Results (Dendrogram)

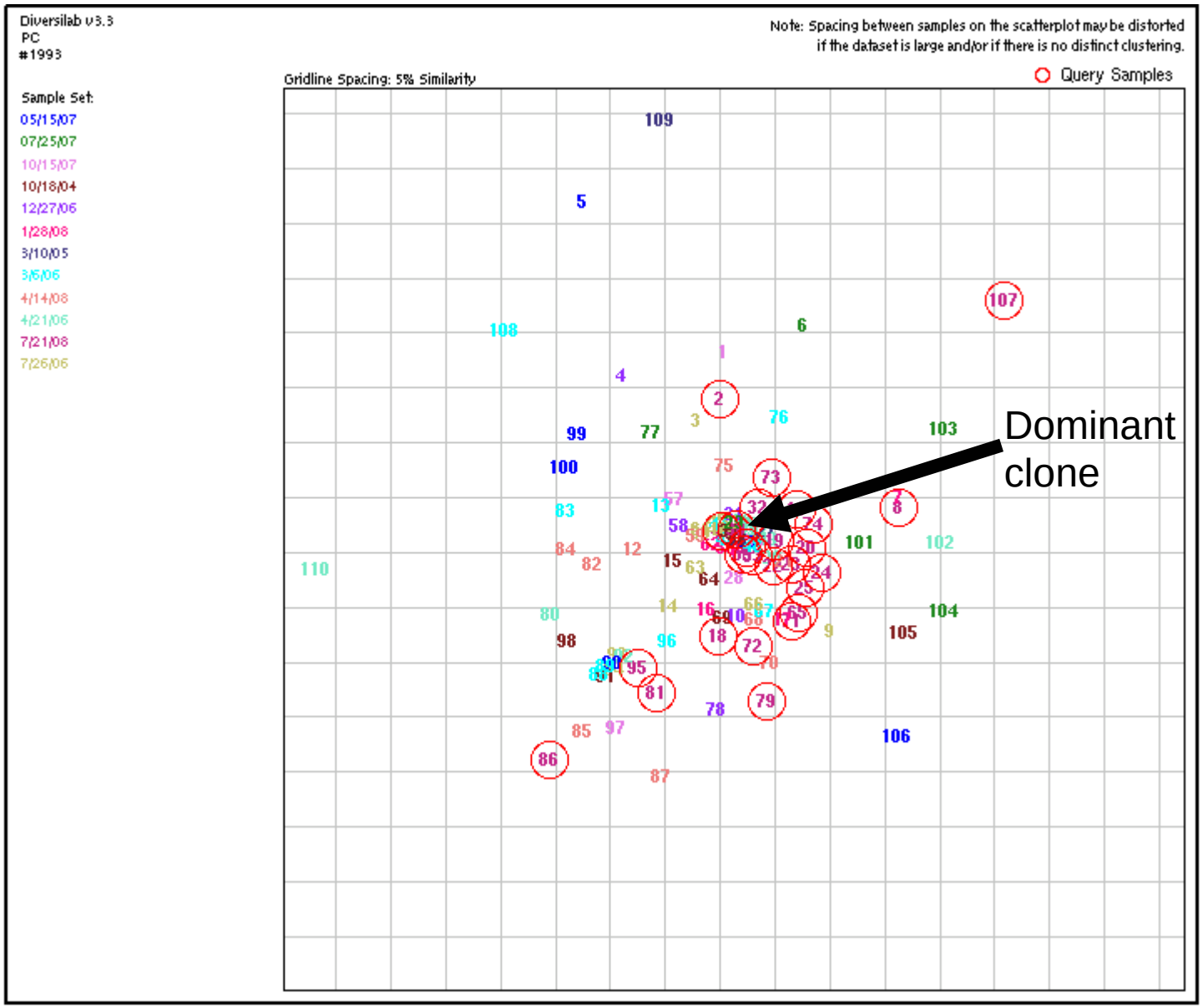


Multi-Drug Resistant *P. aeruginosa* Clusters by Year



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

July 2008 Scatterplot



DiversiLab Report

Newly submitted *Enterobacter cloacae* isolates XXXX and XXXXX 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 simiar to isolates XXXX and XXXXX.

Newly submitted isolates XXXXX 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 XXXXX, XXXX,and XXXXX are similar to previously submitted isolate XXXXXX. Newly submitted isolate XXXXX is clearly distinguishable from from all isolates in the library.

Molecular Typing Redefined Infection Control in CF Center

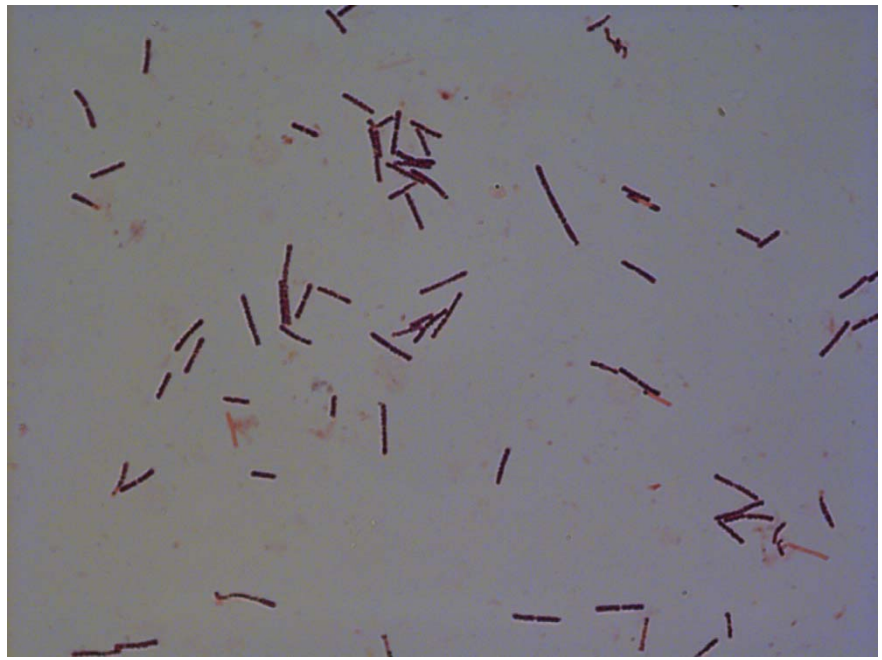
- Dominant MRPA clone was focus of infection control efforts
  - Facile transmission between patients
- Children were tracked, treated promptly, and placed in contact isolation at TCH
- Parents were counseled ; multiple interventions
- As of July 2008, only 2 new patients were identified with the dominant 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



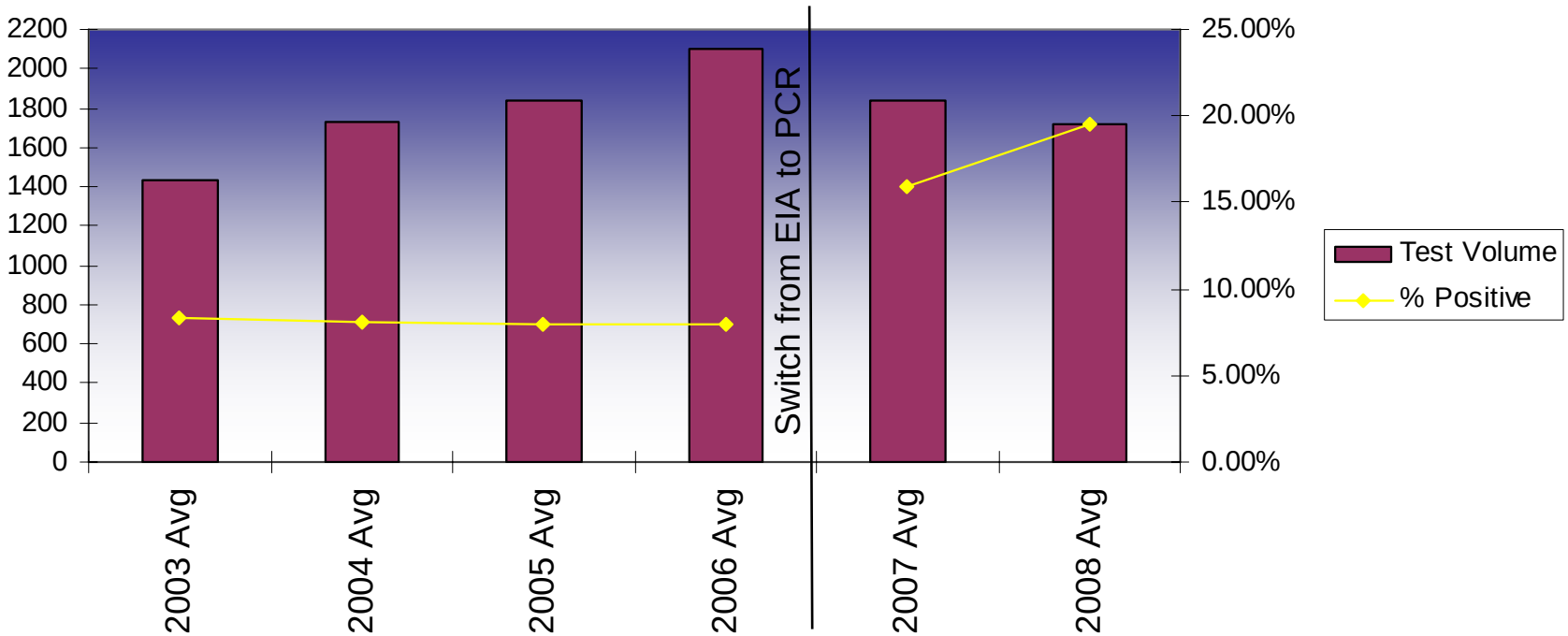
Gram stain

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

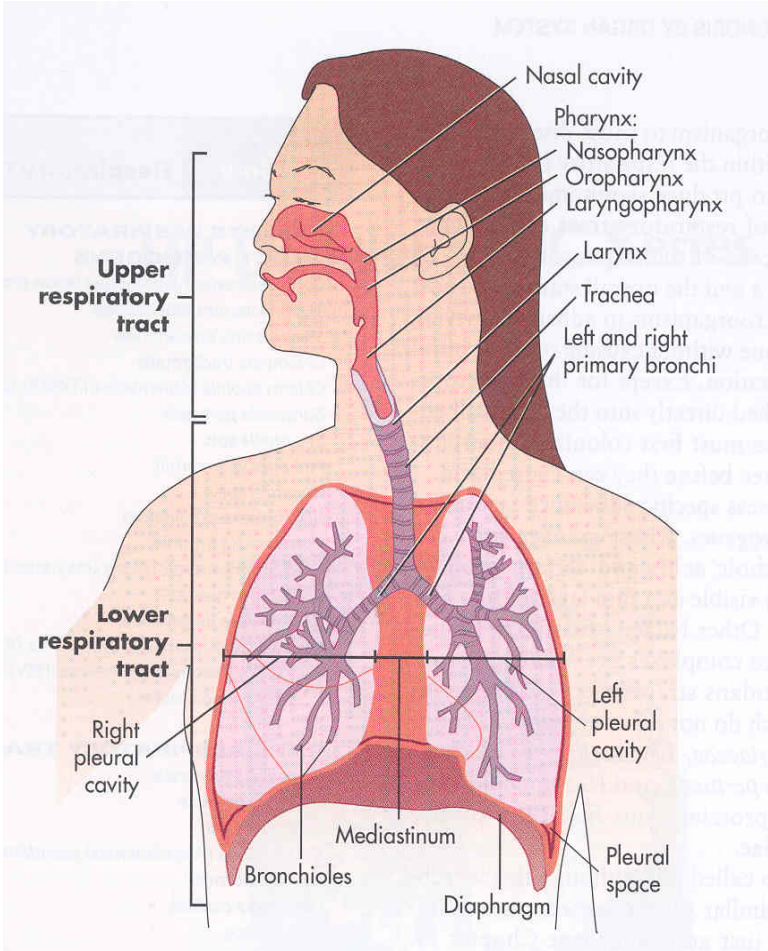
Negative for toxigenic Clostridium difficile.

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.

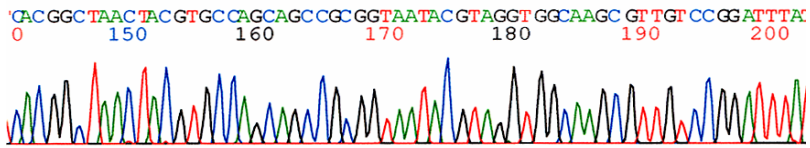
Methodology: Bacterial DNA, if present, was extracted from a stool specimen. Real time PCR with primers and probes specific for *Clostridium difficile* toxin producing genes tcdA and tcdB was performed. This test was developed and its performance characteristics determined by Texas Children's Hospital. 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

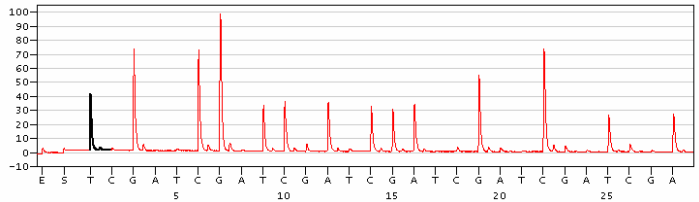


BACTERIAL/FUNGAL IDENTIFICATION



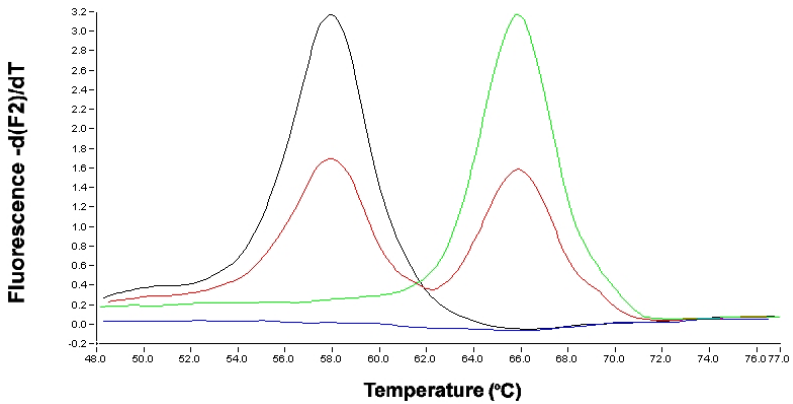
DNA SEQUENCING

Today



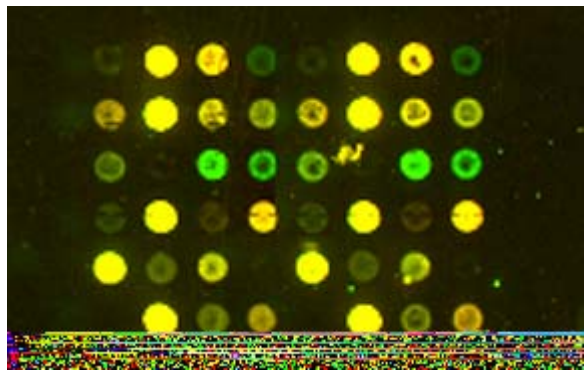
PYROSEQUENCING

Today



REAL-TIME PCR

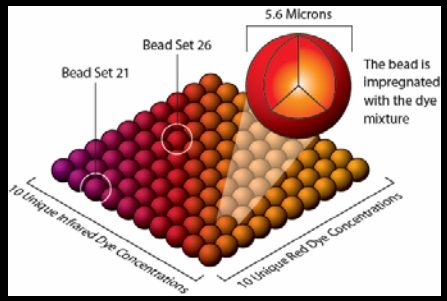
Today



ARRAYS AND MICROARRAYS

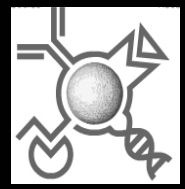
Have arrived

Liquid Bead Arrays



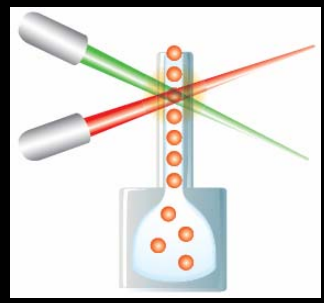
Antibodies

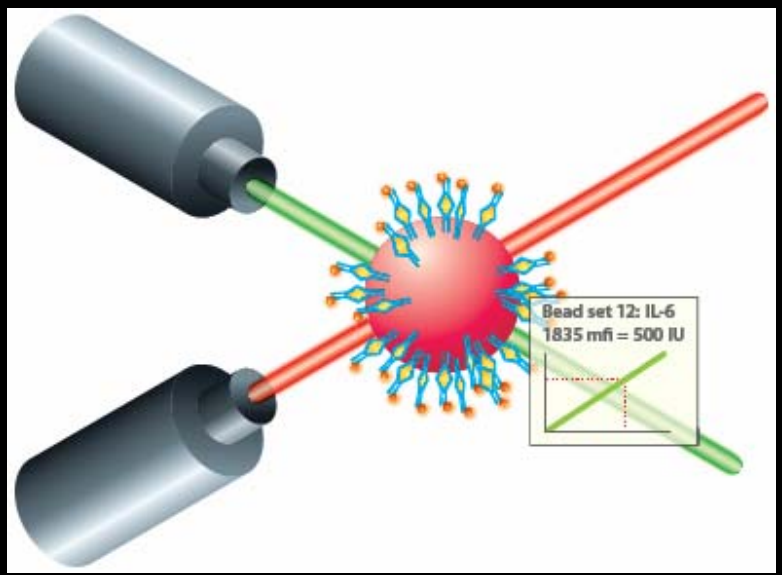
Proteins

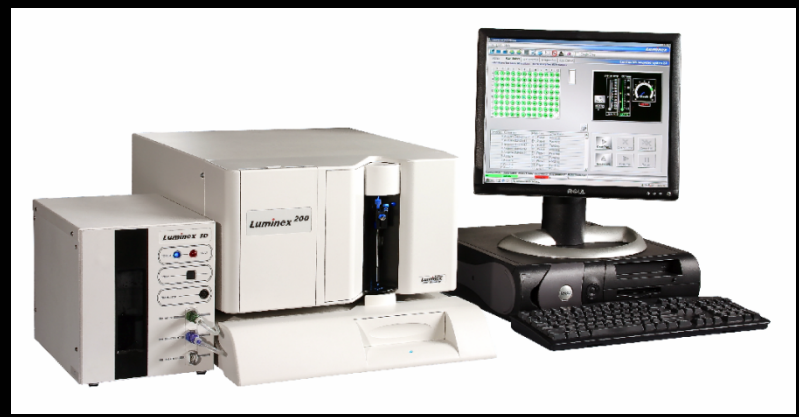


Peptides

Oligonucleotides







Luminex-100

Panomics Inc.

JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2007, p. 2626–2634 Vol. 45, No. 8
0095-1137/07/$08.00+0 doi:10.1128/JCM.02501-06

High-Throughput, Sensitive, and Accurate Multiplex PCR-Microsphere Flow Cytometry System for Large-Scale Comprehensive Detection of Respiratory Viruses▽†

Wai-Ming Lee,[1]* Kris Grindle,[1] Tressa Pappas,[1] David J. Marshall,[3] Michael J. Moser,[3] Edward L. Beaty,[3] Peter A. Shult,[2] James R. Prudent,[3] and James E. Gern[1]

Department of Pediatrics and Medicine[1] and Wisconsin State Laboratory of Hygiene,[2] University of Wisconsin, Madison, Wisconsin, and EraGen Biosciences Incorporated, Madison, Wisconsin[3]

Received 13 December 2006/Returned for modification 5 March 2007/Accepted 20 April 2007

Respiratory viral panel (Luminex) was FDA-approved on Jan. 3, 2008

JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2007, p. 2965–2970 Vol. 45, No. 9
0095-1137/07/$08.00+0 doi:10.1128/JCM.02436-06

Development of a Respiratory Virus Panel Test for Detection of Twenty Human Respiratory Viruses by Use of Multiplex PCR and a Fluid Microbead-Based Assay▽

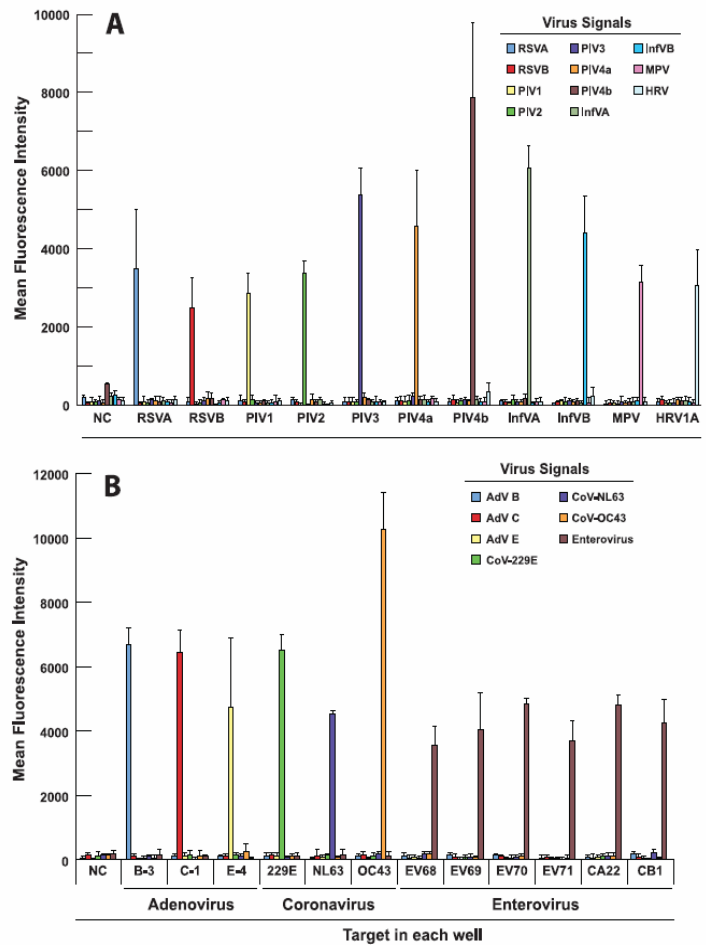
J. Mahony,[1]* S. Chong,[1] F. Merante,[2] S. Yaghoubian,[2] T. Sinha,[1] C. Lisle,[2] and R. Janeczko[2]

Department of Pathology and Molecular Medicine, McMaster University, and St. Joseph's Healthcare, Hamilton, Ontario, Canada,[1] and TmBioscience Corporation, Toronto, Ontario, Canada[2]

Received 4 December 2006/Returned for modification 4 April 2007/Accepted 17 June 2007

Array-Based Respiratory Virus Detection

- Liquid bead arrays by Respiratory Multi-code-PLx Assay (RMA) and microsphere flow cytometery (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



Lee W-M *et al*. (2007) J Clin Microbiol 45:2626-2634

RVP Report Text from ViraCor -1

RESP VIRAL PANEL

NASAL WASH Results as Follows:

Metapneumovirus	Not Detected
Rhinovirus	POSITIVE
Influenza A	Not Detected
Influenza A subtype H1	Not Detected
Influenza A subtype H3	Not Detected
Influenza B	Not Detected
RSV A	Not Detected
RSV B	Not Detected
Parainfluenza 1	Not Detected
Parainfluenza 2	Not Detected
Parainfluenza 3	Not Detected
Adenovirus	Not Detected

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.

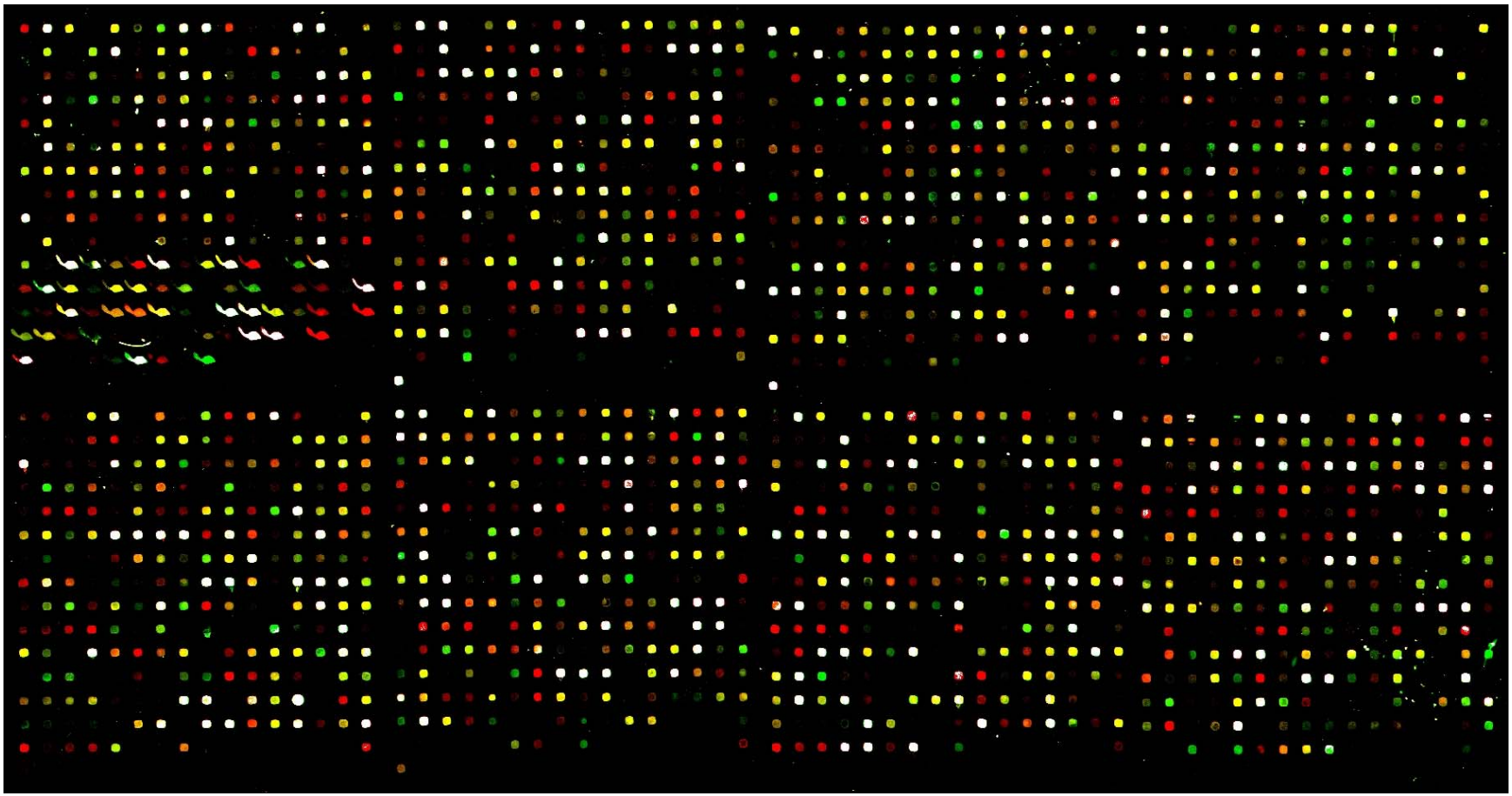
The performance characteristics for this specimen are unknown. This specimen type has not been cleared by the FDA. Results should be used in conjunction with clinical findings.

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

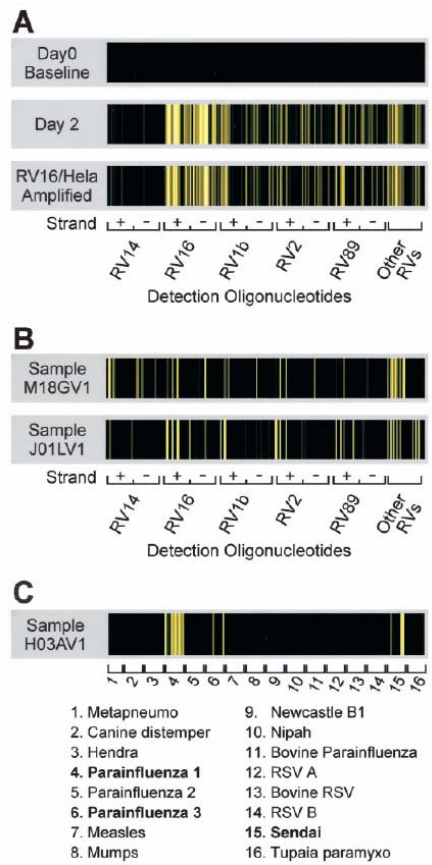
Viracor

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



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0095-1137/07/$08.00+0 doi:10.1128/JCM.02187-06

Loss of Bacterial Diversity during Antibiotic Treatment of Intubated Patients Colonized with *Pseudomonas aeruginosa*▽

J. L. Flanagan,[1]† E. L. Brodie,[2]† L. Weng,[3] S. V. Lynch,[1] O. Garcia,[1] R. Brown,[1] P. Hugenholtz,[3] T. Z. DeSantis,[2] G. L. Andersen,[2] J. P. Wiener-Kronish,[1] and J. Bristow[3]*

Department of Anesthesia and Perioperative Care, University of California, San Francisco, California 94143[1]; Earth Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, California 94720[2]; and DOE Joint Genome Institute, 2800 Mitchell Drive, Bldg. 400-404, Walnut Creek, California 94598[3]

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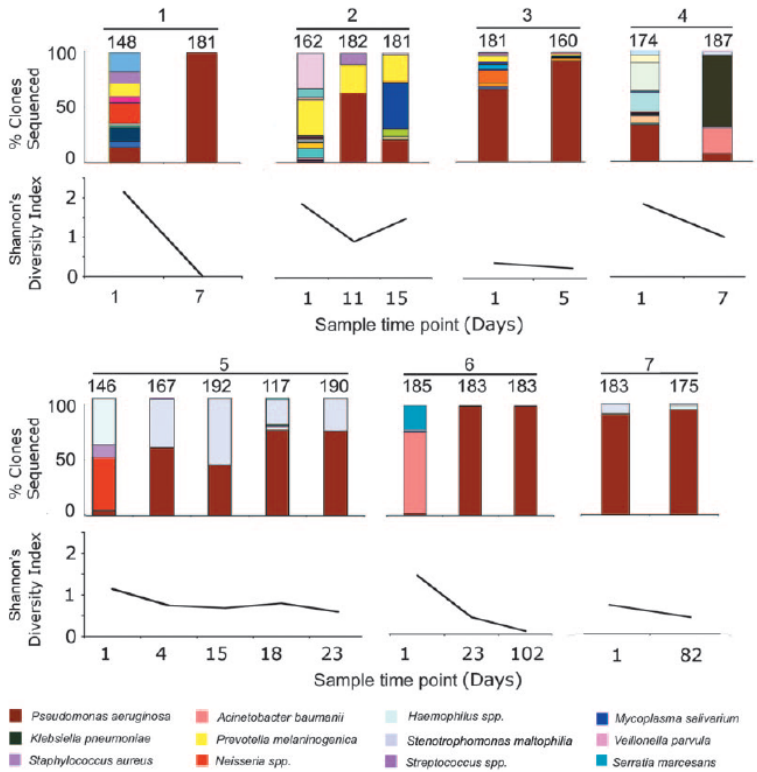
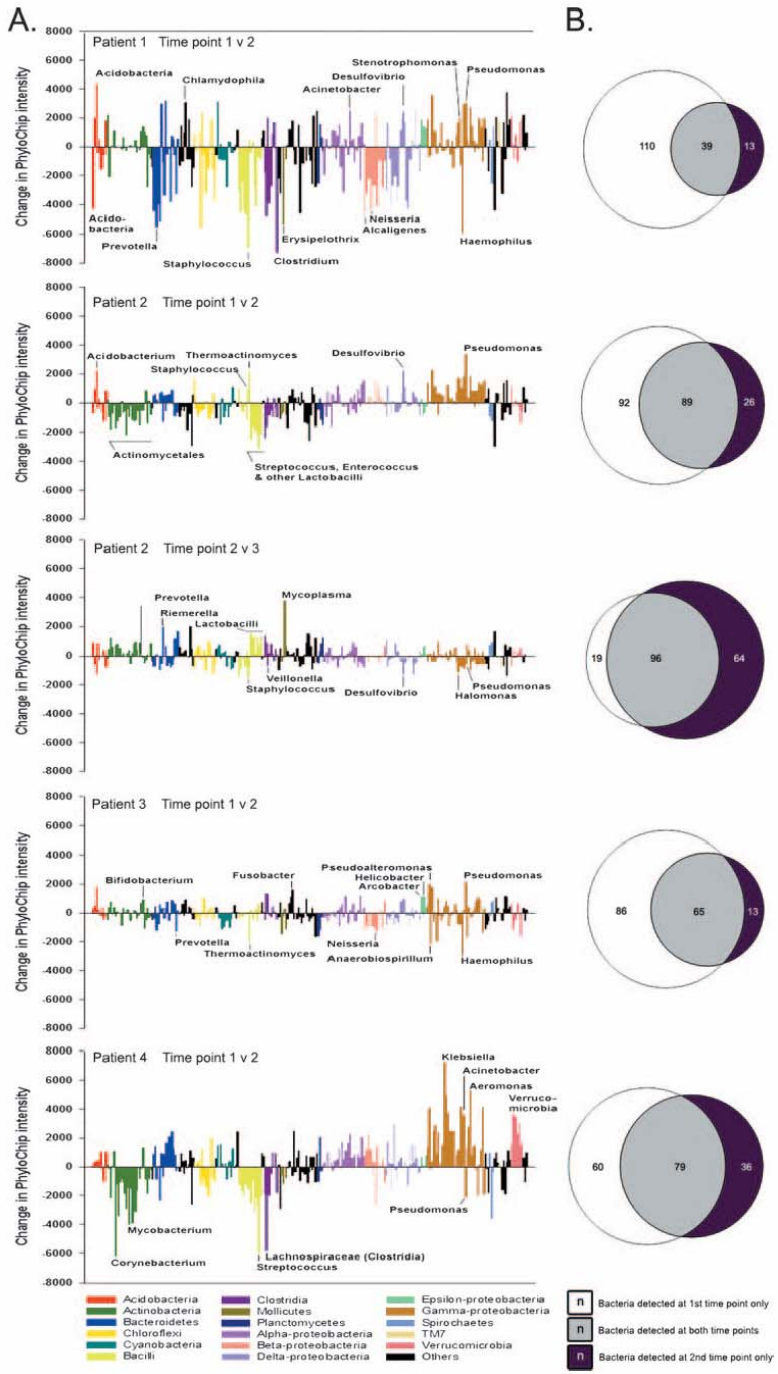
- **PhyloChip – high-density oligonucleotide microarray for bacterial detection**
- **More than 8,000 taxa / chip**
  - **At least 11 probes per taxon**
- **16S rRNA gene sequencing detected reduction from 16.2 to 5.6 (mean number of) bacterial species with antibiotic therapy**



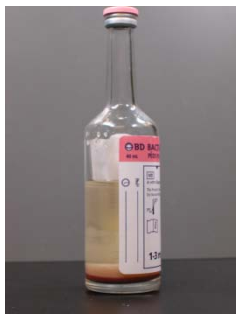
- Bacterial PhyloChip studies in human endotracheal aspirates
  - intubated ICU patients
- Sampling at beginning of parenteral antibiotic versus 4-10 days of therapy
- **Loss of bacterial diversity was correlated with ventilator-associated pneumonia during antibiotic therapy**

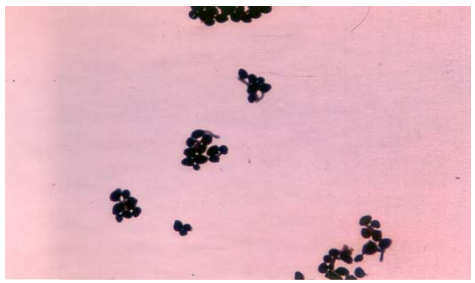


Flanagan JL et al. (2007) J Clin Microbiol 45:1954-1962.

From Microbial Genomics to Diagnostic Microbiology : BENCH TO BENCH

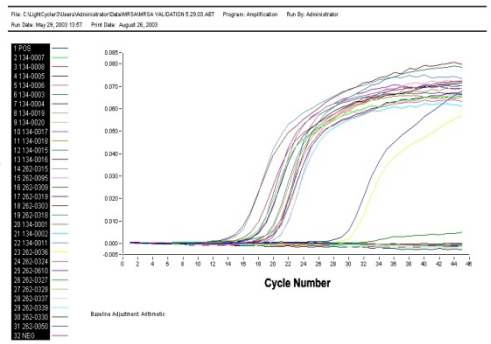
- **POSITIVE CULTURES**

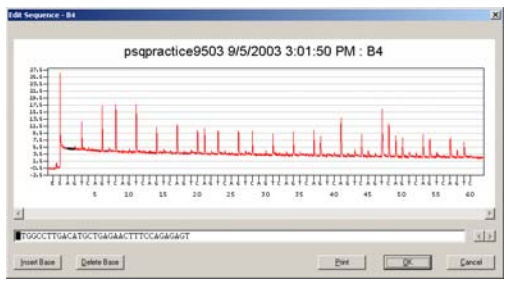




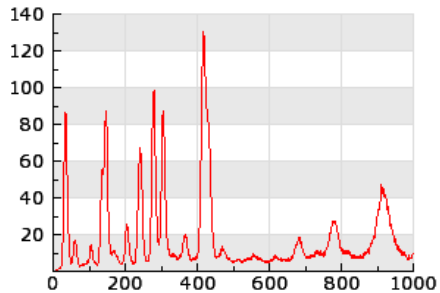
Gram Stain

Real-Time PCR

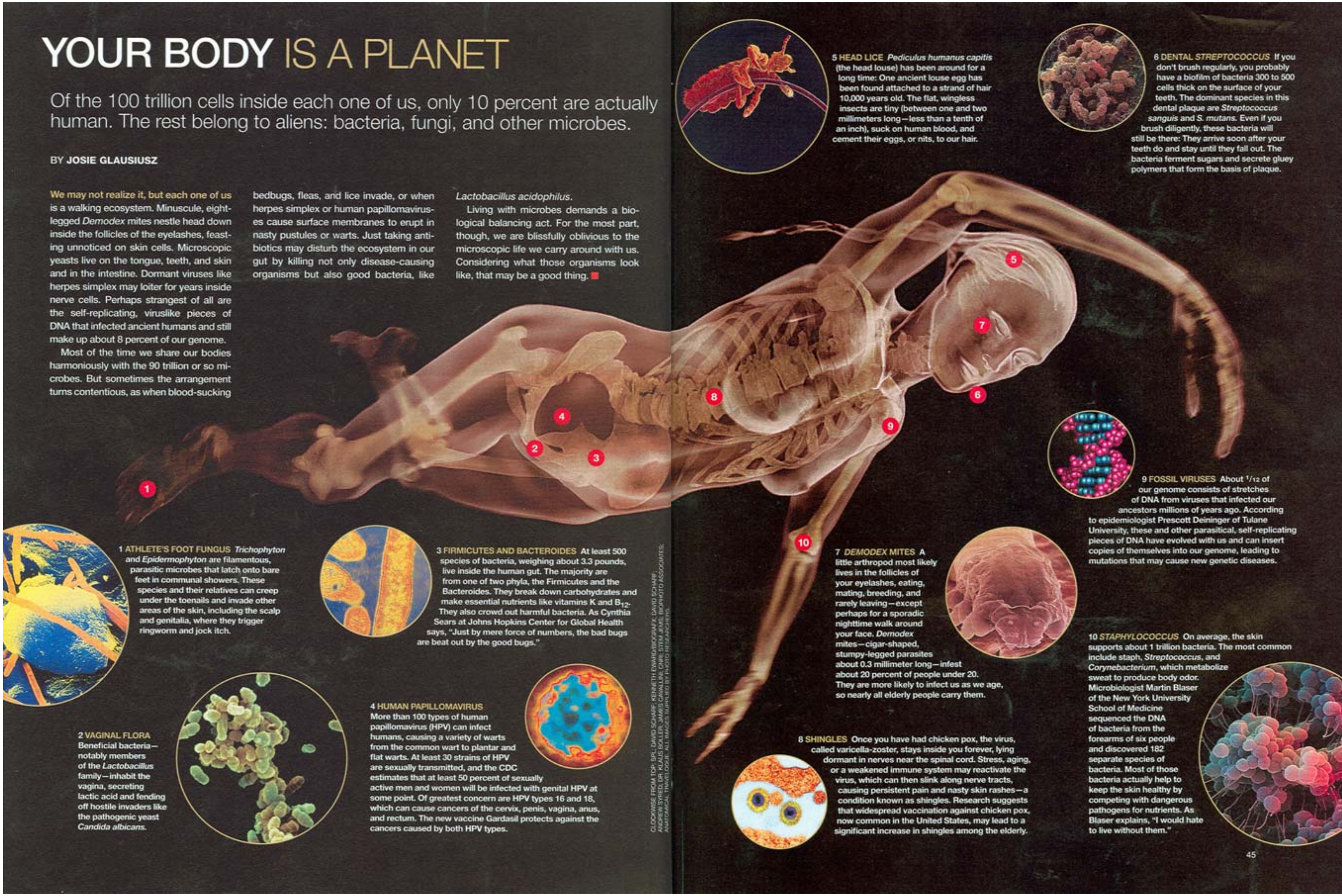




Pyrosequencing



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 GLAUSIUSZ**

We may not realize it, but each one of us is a walking ecosystem. Minuscule, eight-legged *Demodex* mites nestle head down inside the follicles of the eyelashes, feasting unnoticed on skin cells. Microscopic yeasts live on the tongue, teeth, and skin and in the intestine. Dormant viruses like herpes simplex may loiter for years inside nerve cells. Perhaps strangest of all are the self-replicating, viruslike pieces of DNA that infected ancient humans and still make up about 8 percent of our genome.

Most of the time we share our bodies harmoniously with the 90 trillion or so microbes. But sometimes the arrangement turns contentious, as when blood-sucking bedbugs, fleas, and lice invade, or when herpes simplex or human papillomaviruses cause surface membranes to erupt in nasty pustules or warts. Just taking antibiotics may disturb the ecosystem in our gut by killing not only disease-causing organisms but also good bacteria, like *Lactobacillus acidophilus*.

Living with microbes demands a biological balancing act. For the most part, though, we are blissfully oblivious to the microscopic life we carry around with us. Considering what those organisms look like, that may be a good thing. ■

1 ATHLETE'S FOOT FUNGUS *Trichophyton* and *Epidermophyton* are filamentous, parasitic microbes that latch onto bare feet in communal showers. These species and their relatives can creep under the toenails and invade other areas of the skin, including the scalp and genitalia, where they trigger ringworm and jock itch.

2 VAGINAL FLORA Beneficial bacteria—notably members of the *Lactobacillus* family—inhabit the vagina, secreting lactic acid and fending off hostile invaders like the pathogenic yeast *Candida albicans*.

3 FIRMICUTES AND BACTEROIDES At least 500 species of bacteria, weighing about 3.3 pounds, live inside the human gut. The majority are from one of two phyla, the Firmicutes and the Bacteroides. They break down carbohydrates and make essential nutrients like vitamins K and B_{12}. They also crowd out harmful bacteria. As Cynthia Sears at Johns Hopkins Center for Global Health says, "Just by mere force of numbers, the bad bugs are beat out by the good bugs."

4 HUMAN PAPILLOMAVIRUS More than 100 types of human papillomavirus (HPV) can infect humans, causing a variety of warts from the common wart to plantar and flat warts. At least 30 strains of HPV are sexually transmitted, and the CDC estimates that at least 50 percent of sexually active men and women will be infected with genital HPV at some point. Of greatest concern are HPV types 16 and 18, which can cause cancers of the cervix, penis, vagina, anus, and rectum. The new vaccine Gardasil protects against the cancers caused by both HPV types.

5 HEAD LICE *Pediculus humanus capitis* (the head louse) has been around for a long time: One ancient louse egg has been found attached to a strand of hair 10,000 years old. The flat, wingless insects are tiny (between one and two millimeters long—less than a tenth of an inch), suck on human blood, and cement their eggs, or nits, to our hair.

6 DENTAL *STREPTOCOCCUS* If you don't brush regularly, you probably have a biofilm of bacteria 300 to 500 cells thick on the surface of your teeth. The dominant species in this dental plaque are *Streptococcus sanguis* and *S. mutans*. Even if you brush diligently, these bacteria will still be there: They arrive soon after your teeth do and stay until they fall out. The bacteria ferment sugars and secrete gluey polymers that form the basis of plaque.

7 *DEMODEX* MITES A little arthropod most likely lives in the follicles of your eyelashes, eating, mating, breeding, and rarely leaving—except perhaps for a sporadic nighttime walk around your face. *Demodex* mites—cigar-shaped, stumpy-legged parasites about 0.3 millimeter long—infest about 20 percent of people under 20. They are more likely to infect us as we age, so nearly all elderly people carry them.

8 SHINGLES Once you have had chicken pox, the virus, called varicella-zoster, stays inside you forever, lying dormant in nerves near the spinal cord. Stress, aging, or a weakened immune system may reactivate the virus, which can then slink along nerve tracts, causing persistent pain and nasty skin rashes—a condition known as shingles. Research suggests that widespread vaccination against chicken pox, now common in the United States, may lead to a significant increase in shingles among the elderly.

9 FOSSIL VIRUSES About 1/12 of our genome consists of stretches of DNA from viruses that infected our ancestors millions of years ago. According to epidemiologist Prescott Deininger of Tulane University, these and other parasitical, self-replicating pieces of DNA have evolved with us and can insert copies of themselves into our genome, leading to mutations that may cause new genetic diseases.

10 *STAPHYLOCOCCUS* On average, the skin supports about 1 trillion bacteria. The most common include staph, *Streptococcus*, and *Corynebacterium*, which metabolize sweat to produce body odor. Microbiologist Martin Blaser of the New York University School of Medicine sequenced the DNA of bacteria from the forearms of six people and discovered 182 separate species of bacteria. Most of those bacteria actually help to keep the skin healthy by competing with dangerous pathogens for nutrients. As Blaser explains, "I would hate to live without them."

45

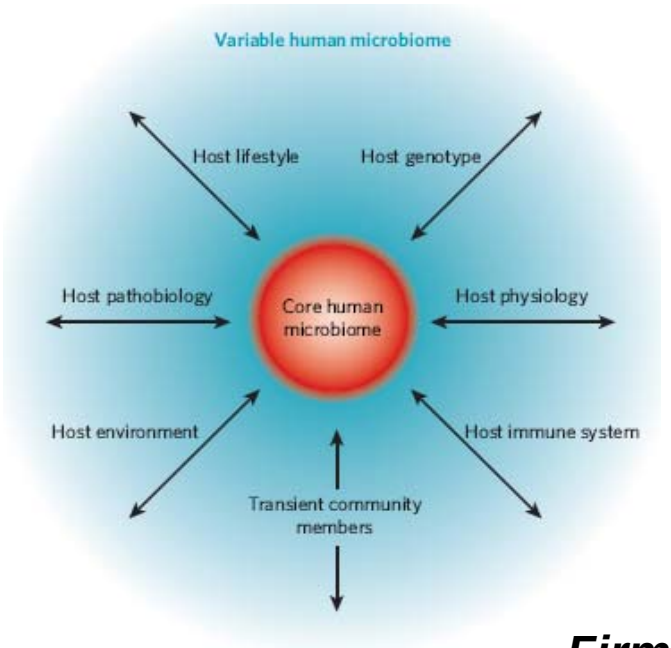
(Glausiusz J. *Discover* June 2007)

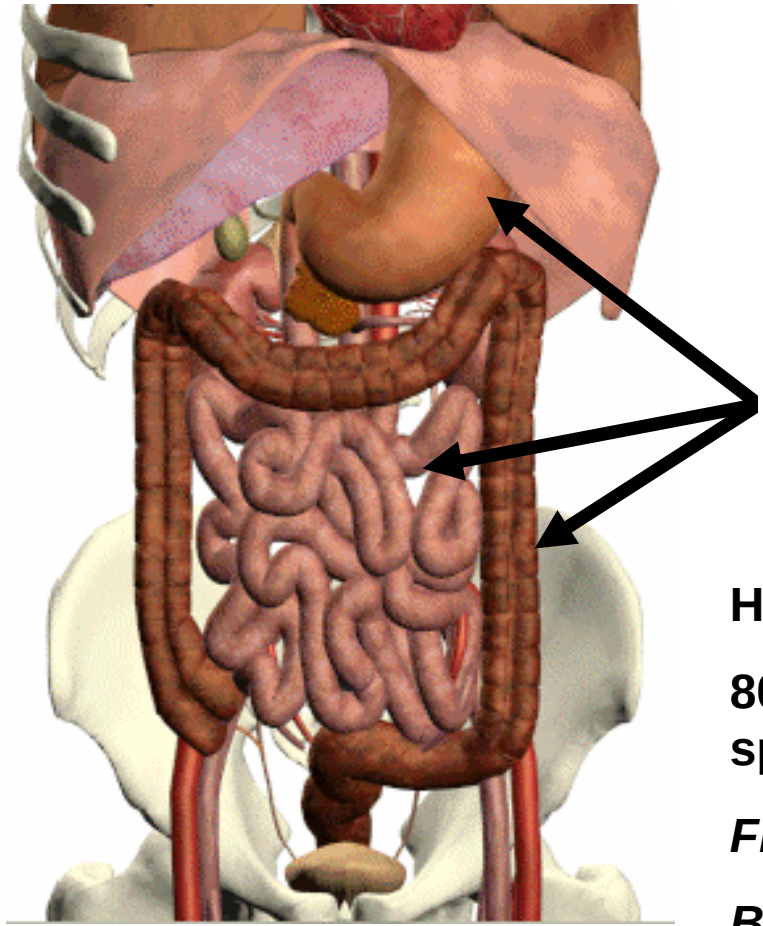
The Human Microbiome Project: Indigenous Microbiota and Microbiome

P Eckburg et al. *Science (*2005) 308:1635-1638

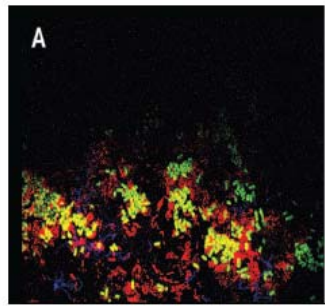
>60% novel bacteria

>80% nonculturable bacteria





Mixed Microbial Communities



Human Colon

800-1000 species

Firmicutes

Bacteroidetes

***Firmicutes* include *Lactobacillus* spp.**

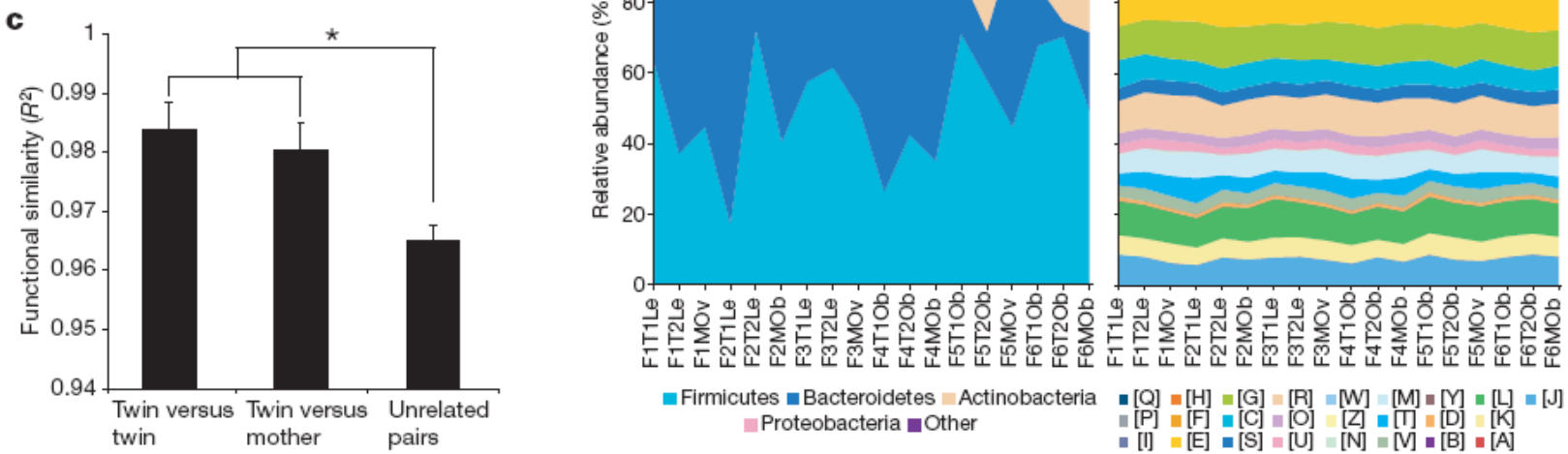
PJ Turnbaugh et al. *Nature* (2007) 449:804-810.

S. Macfarlane et al. *Appl Environ Microbiol* (2005) 71:7483-7492.

Is it Microbial Composition or Functional Genomics?

A core gut microbiome in obese and lean twins

Peter J. Turnbaugh[1], Micah Hamady[3], Tanya Yatsunenko[1], Brandi L. Cantarel[5], Alexis Duncan[2], Ruth E. Ley[1], Mitchell L. Sogin[6], William J. Jones[7], Bruce A. Roe[8], Jason P. Affourtit[9], Michael Egholm[9], Bernard Henrissat[5], Andrew C. Heath[2], Rob Knight[4] & Jeffrey I. Gordon[1]



Turnbaugh et al. 2009;457:480-485.

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