The Molecular Epidemiology of Tuberculosis

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Airborne pathogen, *Mycobacterium tuberculosis*

- Slow grower; doubles 24hrs; 3-4 weeks to culture
- 3-4 weeks for susceptibility testing
- Highly transmissible; requires BL3 facilities

TB Statistics
- 2 billion infected (1/3 world population)
- 8-9 million new cases each year
- 1.6 million deaths per year (25% of all preventable deaths)
- This means 4,000 deaths each day, a death every 20 seconds
- 85% of the mortality in developing countries

2008 WHO report on TB
HIV and Multidrug Resistance

- Co-infection of M. tb and HIV a deadly duet
- 11% co-infected (range from 1% to over 60%)
- Reactivation of tuberculosis or rapid progression to disease are markers for HIV

Multidrug Resistance (INH & RIF)
- Multidrug resistance is emerging in virtually every country
- 425,000 new MDR cases annually
- Estimated 50 million infected with MDR

The last TB-specific antibiotic, ethambutol, was discovered in 1968!

- Streptomycin
- PAS
- Thiacetazone
- Isoniazid
- Pyrazinamide
- Kanamycin/Amikacin
- Ethionamide
- Capreomycin
- Rifampin
- Ethambutol

FIRST LINE AGENTS ARE IN BOXES

Molecular Epidemiology

- Hybrid field incorporating molecular biology, epidemiology and clinical medicine
- Track strains in populations to indicate/refute transmission (e.g., outbreaks)

Molecular Epidemiology

Local Epidemiology

Are M. tuberculosis isolates recovered from localized cases of disease the same or different strains?

Global Epidemiology

Are strains causing disease in one geographic area related to those isolates world-wide?
Molecular Tools - Genotyping Methods

Primary Genotyping Method
- **IS6110** Southern blot hybridization

Secondary Genotyping Methods
- Spoligotyping Binary typing, DR region
- PGRS Southern blot hybridization
- VNTR, MIRU PCR, multiple targets
- IS6110 mapping Southern blot hybridization
- DNA sequencing Resistance targets, SNP
- Array analysis Deletion mapping

Genotyping Targets to Discriminate *M. tuberculosis*

Insertion Sequence **IS6110**

IS6110 DNA Fingerprinting
- Standardized methodology
- Southern blot hybridization
- PvuII restriction digest
- Common right-side hybridization probe
- Common molecular weight standards
- Digitized patterns
- Pattern matching software
IS610 DNA Fingerprint

DNA Fingerprints of *M. tuberculosis* Strains

Searching the Database for Strain W4
**IS6110 Genotyping**

Limitations:
- Not able to subtype low copy number strains (<6 IS6110 insertions)
- Not able to determine strain relatedness
- Turn-around time is too slow

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**Spoligotyping: A Secondary Typing Method**

- PCR-based, binary hybridization method
- DR-region: 36 bp repeats / 35-41 bp spacers
- Rapid turn-around time, objective data
- Does not provide IS6110-like discrimination
- It’s a “grouper”

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**Genotyping Data – Public Health Issues**

- Evaluate nosocomial and community transmission
- Evaluate suspected cases of laboratory contamination
- Distinguish relapse vs. re-infection
- Genotype drug resistance genes to distinguish spread vs acquisition
- Distinguish recent transmission and endemic strains
SRO Outbreak in San Francisco

Molecular Epidemiology

PHRI TB CENTER

- >27,000 *M. tuberculosis* isolates
- Diverse geography – MDR isolates
- All fingerprinted using IS6110
- 35% spoligotyped
- >2000 sSNP analysis
- Patient and strain database
- Archived fingerprint library

Recurrent tuberculosis

Exogenous Reinfection

*Exogenous reinfection with multidrug-resistant Mycobacterium tuberculosis in patients with advanced HIV infection*

Small, PM, Shafer, RW, Hopewell, PC, Singh, SP, Murphy, MJ, Desmond, E., Sierra, MF, Schoolnik, GK

"P" Strain – MDR Causing Re-Infections in AIDS Patients

Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment

12/16 patients relapsed with exogenous strains (one HIV+ and 15 HIV-)

Tuberculosis in New York City
WHEN AND WHERE

March 1992, 168th Street, NYC

Poverty, homelessness, crowding, substance abuse
HIV/AIDS epidemic
Decline of public health infrastructure
- Marked reduction in TB control program staff and clinic facilities
- Lack of accessible health care
TB abroad on the rise; immigration from high prevalence countries
Poor infection control practices in hospitals
Poor treatment practices
- No susceptibility results for most patients
- Bad regimens
By 1989, less than half of patients who began treatment were cured

Figure 1: Tuberculosis Cases and Rates, New York City, 1970-1998

Causes of Resurgent Tuberculosis in NYC
W Strain MDR Outbreak in NYC

- January 1990 - August 1993
- 43 months - 8,021 cases
- 357 patients with W strain tuberculosis
- Spread in NYC hospitals and state prisons
- All resistant to first line drugs
- 86% HIV infected; >90% mortality
- 160 patients identified since study
- 22 patients identified outside of NYC

Bifani et al., JAMA 1996:275:452
Munsiff et al., JID 2003:188:356

W MDR Outbreak: 1990-1993

- Isoniazid (100%)
  - katG - 315:AGC>ACA; Ser>Thr
- Rifampin (100%)
  - rpoB - 526:CAC>TAC; His>Tyr
- Streptomycin (100%)
  - rpsL - 43:AAG>AGC; Lys>Arg
- Ethambutol (100%)
  - embB - 306:ATG>GTG; Met>Val
- Pyrazinamide (55%)
- Kanamycin (92%)
- Fluoroquinolones (0%)

Bifani et al., JAMA 1996:275:452
Munsiff et al., JID 2003:188:356

Creating Extremely Drug Resistant W Strains


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<tr>
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<tbody>
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<td>Capreomycin</td>
<td>204</td>
<td>10 (5)</td>
<td>151</td>
<td>24 (16)</td>
<td>.001</td>
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<tr>
<td>Ciprofloxacin</td>
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<td>2 (1)</td>
<td>152</td>
<td>2 (1)</td>
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<td>163</td>
<td>42 (27)</td>
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<td>Fluoroquinolone</td>
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<td>0</td>
<td>153</td>
<td>9 (6)</td>
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<tr>
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<td>147</td>
<td>2 (1)</td>
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<tr>
<td>Pyrazinamide</td>
<td>188</td>
<td>104 (55)</td>
<td>146</td>
<td>68 (46)</td>
<td>.001</td>
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</table>

NOTE: By definition, all strains were resistant to isoniazid, rifampin, ethambutol, and streptomycin.

Munsiff et al., JID 2003:188:356
Lessons Learned and Unlearned

- Infection control essential
- Rapid diagnostic and 2nd line susceptibility testing
- Ensure adequate supply of drugs
- New drugs and more effective regimens needed

REBUILDING NYC TB CONTROL – $1,000,000,000

XDR-TB Outbreak in an HIV-positive Population in South Africa

Jan 2005 – March 2006
1,539 TB diagnosed
542 culture positive cases
168 MDR cases
53 XDR cases
52 / 53 died
44 patients tested: All HIV+

MEDIAN SURVIVAL OF 16 DAYS FROM THE TIME OF DIAGNOSIS

Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa

XDR-TB Outbreak in an HIV-positive Population in South Africa
Drug Resistant *M. tuberculosis*

- Multidrug Resistance (MDR)
  Resistance to at least isoniazid (INH) and rifampin (RIF)
- Multidrug Resistance (Plus)
  Resistance to at least isoniazid and rifampin plus resistance to fluoroquinolones
- Extensively Drug Resistance (XDR)
  Resistance to at least isoniazid and rifampin plus resistance to fluoroquinolones AND one of the second line injectable aminoglycoside drugs (amikacin, kanamycin or capreomycin)

Cure Rates for MDR-TB and XDR-TB

- MDR-TB cure rate 1993-8: 94%
  (fluoroquinolones & surgery were critical variables)
- MDR*-TB cure rate 1993-8: 60%
- XDR-TB cure rate 1993-8: 20%

Case studies from National Jewish TB Center, Denver, CO

Laboratory Contamination of *Mycobacterium tuberculosis* Cultures in New Jersey

- Lane 1 - Patient JH (5) – 12-17-04
- Lane 2 - Patient TA (4) – 12-17-04
- Lane 3 - Patient WS (3) – 12-17-04
  Lane 4 - Patient WS (3) – 9-07-04
  Lane 5 - Patient LH (2) – 12-17-04
  Lane 6 - Patient LH (2) – 7-01-03
- Lane 7 - Patient LH (2) – 11-03-04
- Lane 8 - Patient PS (1) – 12-17-04
  Lane 9 - Patient PS (1) – 7-01-03
**Patient JH: Image # 1**

- JH - 51 y/o male
- 12-15-04 – referred to a local NJ chest clinic with symptoms of TB.
- Sputum sample was 4+ on smear for AFB and grew *M.tb* that was resistant to INH and SM
- 12-17-04 – DOT started with RIPE, daily
  - However he did not complete his treatment in NJ
- 1-26-05 – patient relocated to Orange County, California and in February ’05 moved to Illinois

**Patient JH: Bacteriology**

<table>
<thead>
<tr>
<th>Date</th>
<th>Smear</th>
<th>Culture</th>
<th>Specimen</th>
<th>Sp#</th>
<th>Resistance</th>
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<td>4+</td>
<td><em>M.tb</em></td>
<td>Sputum</td>
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<tr>
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<td>&gt;10</td>
<td><em>M.tb</em></td>
<td>Sputum</td>
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<td>INH, SM</td>
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<td>1-5</td>
<td><em>M.tb</em></td>
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<tr>
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<td>Neg.</td>
<td><em>M.tb</em></td>
<td>Sputum</td>
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<tr>
<td>1-12-05</td>
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<td><em>M.tb</em></td>
<td>Sputum</td>
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<tr>
<td>1-14-05</td>
<td>Neg.</td>
<td><em>M.tb</em></td>
<td>Sputum</td>
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</tbody>
</table>

**Patient LH: Images # 5, 6 and 7**

- LH – 42 y/o diabetic H/F born in DR and immigrated to the US in Feb. 1986
- Sept. 2004 – she c/o wt. loss and cough
- 10-31-04 – 11-15-04 – patient was admitted to a local hospital where she was diagnosed with pulmonary TB. A CXR revealed cavitary infiltrate in the RUL consistent with active TB
- 11-5-04 – DOT started with RIPE, daily for 8 weeks followed by RI, daily for 16 weeks.
- Her sputum was initially thought to convert to negative on culture for *M.tb* at 4 weeks
- 12-17-04 – sputum specimen collected and processed and on 12-21-04 the lab grew *M.tb*. This sequence of events, if true, could have caused an extension of treatment

**Patient LH: Bacteriology**

<table>
<thead>
<tr>
<th>Date</th>
<th>Smear</th>
<th>Culture</th>
<th>Specimen</th>
<th>Sp#</th>
<th>Sensitivity</th>
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<tr>
<td>11-3-04</td>
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<td>Sputum</td>
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<td>+</td>
<td><em>M.tb</em></td>
<td>Sputum</td>
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<td>11-11-04</td>
<td>+</td>
<td><em>M.tb</em></td>
<td>Sputum</td>
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<tr>
<td>11-16-04</td>
<td>Neg.</td>
<td><em>M.tb</em></td>
<td>Sputum</td>
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<tr>
<td>12-17-04</td>
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<td><em>M.tb</em></td>
<td>Sputum</td>
<td>S12-170-21</td>
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<tr>
<td>1-12-05</td>
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<td>Neg.</td>
<td>Sputum</td>
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</tr>
<tr>
<td>2-16-04</td>
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<td>Sputum</td>
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<tr>
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<td>Neg.</td>
<td>Sputum</td>
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</table>
Patient PS: Images # 8 & 9

- PS – 32 y/o H/M born in Ecuador and immigrated to the US in June 2002
- May 2003 – admitted to hospital with c/o cough, fever, night sweats, wt. loss, and hemoptysis
- A chest CT scan revealed LUL cavitary lesions associated with pleural thickening and fibrotic changes
- Sputum smears were + for AFB and cultures grew pan-susceptible M.tb
- 7-8-03 – DOT started with RIPE, daily for 8 weeks, followed by RI, BIW for 18 weeks

- Sputum converted to negative on smear and culture for M.tb at 6 weeks
- 1-9-04 – DOT was discontinued (26 weeks) when RI were stopped. The patient felt well and had a normal ESR (8 mm/hr) when treatment was stopped
- 7-16-04 – he was seen in clinic for routine f/u visit and remained well; his sputum was smear and culture negative for AFB
- 12-17-04 – patient presented for routine clinic visit, felt well and was w/o complaints. A sputum sample (S12-0172-72) collected at this visit grew M.tb on culture

Case Study

Background - 1

- 8/5/05 patient KD admitted to hospital “A” with diagnosis of suspected pulmonary tuberculosis
  - CXR abnormal/non-cavitary
  - Sputum smear positive (4+), final culture M.tb
  - Resistant INH
- 8/10/05 patient threatens to leave hospital AMA
  - Attempted problem identification/problem resolution
  - No health officer restraining order requested
  - 1:1 monitoring in place
- 8/11/05 patient leaves hospital AMA
  - Numerous attempts to locate patient prove futile
  - Patient provided fictitious identity and locating information to hospital
  - Cell phone conversation with index patient indicates “on way to Florida” with pregnant girlfriend
  - Case dispositioned as lost to follow-up
Background - 2

- 4/5/06 patient CB admitted to hospital “B” with diagnosis of suspected pulmonary tuberculosis
  - CXR abnormal/non-cavitary
  - Sputum smear positive (4+), final culture MTB
  - Resistant INH, EMB, and PZA
- 4/6/06 patient threatens to leave hospital AMA
  - Attempted problem identification/problem resolution
  - Patient uncooperative
  - Legal intervention requested
    - Health Officer hospital restraining order served
  - Despite completely different demographic information subtle physical similarities existed between patients KD and CB
  - CB denies any knowledge of KD
- Upon hospital discharge CB placed on DOT and followed up as an outpatient at local TB clinic where he completed treatment

Laboratory Contamination

10/1/10: patient MJ, 12 month old infant brought to clinic with respiratory distress
  - X-ray abnormal; suspected pneumonia
  - Sent for BAL to check for a foreign object
  - No foreign objects; BAL cultured
  - Infant hospitalized; treated for pneumonia
10/6/10: infant sent home and symptoms resolved in 2 weeks
10/30/10: laboratory report BAL was culture positive for *M. tuberculosis*

11/4/10: Infant and parents advised of the tuberculosis finding and the baby, although well, was placed on 4 TB drugs IREZ
  - Case reported to the NJ DOH
  - Infant to treated for 6-9 months
  - Monthly clinic visits
  - Contact investigation
11/26/10: *M. tb* fingerprint identified the strain as H37Ra – the laboratory control strain used in susceptibility testing. The laboratory contaminated the BAL. The infant does not have tuberculosis.
11/27/10: Infant taken off drugs; case removed; no investigation
Drug Susceptibility Testing

Current Therapy for Tuberculosis Disease

2HRZE/4HR
- Induction phase: 2 months isoniazid, rifampin, pyrazinamide, ethambutol
- Continuation phase: 4 months isoniazid, rifampin

First Line Drugs
- Streptomycin
- Isoniazid
- Rifampin
- Ethambutol
- Pyrazinamide

Second Line Drugs
- Ethionamide
- Amikacin / Kanamycin
- Capreomycin
- Fluoroquinolones
- PAS
- Cycloserine

Antimycobacterial Agents

METHODS
- Middlebrook 7H10 or 7H11 agar – incorporate drug
- Middlebrook 7H10 or 7H11 agar - E-test
- Lowenstein-Jensen slants
- BACTEC 460 – radioactive CO2
- BACTEC MGIT 960 – oxygen consumption

LIMITATIONS
- Susceptibility requires a growing culture – 3 weeks
- First-line drugs tested first – 3 weeks
- Second-line drugs tested for MDR – 3 weeks
- MDR treatment delayed
It's Just Too Slow!

Advantages
- Speed
- Resistance genes identified
- No synonymous mutations
- Mutation predicts resistance

Challenges
- Bypass culturing
- Infectious & contaminated
- Cost
- Sensitivity / Specificity
- Simplicity
- Flexibility

M. tuberculosis Genome – H37Rv

Genome size (bp): 4,411,532
- 3,959 predicted ORFs (90.8%)
- 2,441 attributed functions
- 912 conserved hypotheticals
- 606 unknowns

Comparative Sequence Analysis
- M. tuberculosis genome is highly conserved
- M. tuberculosis is a monomorphic species
- Synonymous base pair changes are rare
- M. tuberculosis “young” human pathogen
- Non-synonymous changes in drug resistance targets correlate with resistance
Drug Resistance Target Genes

<table>
<thead>
<tr>
<th>Antibiotic agent</th>
<th>Gene</th>
<th>Product</th>
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<tbody>
<tr>
<td>Streptomycin</td>
<td>rpsL</td>
<td>Ribosomal protein S12</td>
</tr>
<tr>
<td>Rifampin</td>
<td>rpoB</td>
<td>β-subunit of RNA polymerase</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>kasG</td>
<td>Catalase-peroxidase</td>
</tr>
<tr>
<td></td>
<td>enoA-pnc</td>
<td>Aldehydreductase</td>
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<tr>
<td></td>
<td>inhA</td>
<td>Enoyl-ACP reductase</td>
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<td>katG</td>
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Fluoroquinolone Resistance and gyrA Mutations

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<tr>
<th>74</th>
<th>GCC . . . . CAC</th>
<th>88</th>
<th>GGC</th>
<th>90</th>
<th>GCG</th>
<th>91</th>
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<tbody>
<tr>
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<td>Gln</td>
<td>His</td>
<td>Ser</td>
<td>Leu</td>
<td>Tyr</td>
<td>Ser</td>
<td>Leu</td>
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<td>Pro</td>
<td>Gly</td>
<td>Ala</td>
<td>Tyr</td>
<td>Asn</td>
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<tr>
<td>(42%)</td>
<td>(15%)</td>
<td>(1%)</td>
<td>(1%)</td>
<td>(3%)</td>
<td>(26%)</td>
<td></td>
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Wild Type: CAC GGC CAC GCG TCG ATC TAC GAC AGC CTG
Resistance: CAC GGC CAC GCG TCG ATC TAC GGC AGC CTG

Rifampin Resistance & rpoB Mutations

All molecular methods to genotype drug resistance in *M. tuberculosis* requires allelic discrimination at the single nucleotide level.
Genotyping Drug Resistance

- Hain reverse hybridization line probe assay (Hain Lifescience, Nehren, Germany)
  - Multiplex PCR and reverse hybridization
  - Identifies major mutations in rpoB, katG and inhA
  - Demonstrated with specimens and culture

- Cepheid (Sunnyvale, CA)
  - PCR and molecular beacon detection
  - Detection of rpoB – surrogate for MDR
  - Closed system with primary specimens

- Abbott’s Ibis, PLEX ID (Abbott Park, IL)
  - Multiplex PCR and mass spectrometry
  - Detection platform able to detect XDR
  - Not evaluated with primary specimens

In Conclusion

- Genotyping drug resistance in *M. tuberculosis* is both rapid and accurate and the consequence is that we are able to dramatically reduce the time to correctly treat an MDR patient and stop the infectious process.

- The refocusing of funds from the development of TB vaccines to implementing rapid diagnostics and developing new drugs for better treatments may be a more effective global approach.