The Molecular Diagnosis of Tuberculosis

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Director, PHRI TB Center
OUTLINE

- TB Statistics
- Biology of *M. tuberculosis*
- Challenges in diagnosing Mtbc
- Diagnosing drug resistance
- Molecular Epidemiology
- Recurrent tuberculosis
- Diagnosing elephant TB
Airborne pathogen, *Mycobacterium tuberculosis*
Highly transmissible; requires BL3 facilities
TB Statistics
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

WHO report on TB
HIV and Tuberculosis

- 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
Pediatric TB Statistics

- 1989 – 1.3 million
- 2000 – 900,000
- 2010 – 1.0 million (3.2% MDR)
- 2014 – 1.0 million, 140,000 deaths
BIOLOGY OF *M. tuberculosis*
Mycobacterium tuberculosis

- Slow grower; doubles 24hrs; 3-4 weeks to culture
- 3-4 weeks for susceptibility testing
- SPEEDING UP THIS PROCESS IS CRITICAL
**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
Genetic Structure of a Given Species

Clonal spectrum

Not clonal (panmictic)
- Helicobacter pylori
  (N. gonorrhoeae)

Somewhat clonal
- Staphylococcus aureus
  (E. coli)

Extremely clonal
- B. anthracis
  (M. tuberculosis)
Comparative Sequence Analysis

- *M. tuberculosis* genome is highly conserved
- *M. tuberculosis* is a monomorphic species
- *M. tuberculosis* lacks extrachromosomal DNA
- *M. tuberculosis* lacks resistance transposons
- Non-synonymous mutations more common than synonymous changes
Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection

Christopher B. Ford\(^1,11\), Philana Ling Lin\(^2,11\), Michael Chase\(^1\), Rupal R. Shah\(^1\), Oleg Iartchouk\(^3\), James Galagan\(^4,5,6\), Nilofar Mohaideen\(^7\), Thomas R. Ioerger\(^8\), James C. Sacchettini\(^7\), Marc Lipsitch\(^1,9\), JoAnne L. Flynn\(^10\), and Sarah M. Fortune\(^1\)

Inferring patient to patient transmission of *Mycobacterium tuberculosis* from whole genome sequencing data

Josephine M Bryant\(^1,1^{*}\), Anita C Schürch\(^2,3,4^{*}\), Henk van Deutekom\(^5\), Simon R Harris\(^1\), Jessica L de Beer\(^2\), Victor de Jager\(^3,6\), Kristin Kremer\(^2\), Sacha A F T van Hijum\(^3,6,7\), Roland J Siezen\(^3,6\), Martien Borgdorff\(^5,8\), Stephen D Bentley\(^1\), Julian Parkhill\(^1^{*}\) and Dick van Soolingen\(^2,9\)

Estimated rate of change: \(~0.3\) SNP per genome per year
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
TB Pipeline is Limited

1944: Streptomycin
1946: PAS
1952: Isoniazid
1955: Pyrazinamide
1956: Thioacetazone
1957: Cycloserine
1965: Kanamycin/amikacin
1966: Rifampin
1967: Ethionamide
1968: Capreomycin
1968: Ethambutol

2013: Bedaquiline approved – first since 1968
Challenges in Diagnosing TB
Challenges in Diagnosing Tuberculosis

- Slow growing bacteria
- Highly infectious
- Diagnosis based on sputum samples
- Pediatrics require alternative sampling
Challenges in Diagnosing Tuberculosis

- Sample acquisition
- Adults: expectorated and induced sputum
- Pediatrics: Gastric aspirate, nasopharyngeal washes, stool samples and string test
Challenges in Diagnosing Tuberculosis

CDC Historical Data: 1993 – 2014

Approximately 25% of pediatric TB cases are culture confirmed for all types of specimens

- Gastric aspirates in children <1
  60-70% culture confirmed

- Gastric aspirates in children > 1
  30-40% culture confirmed
Challenges in Diagnosing Tuberculosis

- Processed sputum – decontaminate with NaOH
- Inoculate to liquid (MGIT) or LJ slant
- 3-4 weeks for growth
- Mtb confirmed by probe
- 3-4 weeks for susceptibility testing
Cepheid GeneXpert: Game Changer
Cepheid GeneXpert

- Direct processing of sputum in closed system
- Molecular beacon detection for Mtb
- 2 hour run time
- 98% detection of smear positive sputum
- ~65% detection of smear negative sputum
- Gates supporting costs in developing countries

Unfortunately alternative samples not as robust
Diagnosing Drug Resistance
Molecular Basis of Drug Resistance

- *M. tuberculosis* genome is highly conserved
- *M. tuberculosis* lacks extrachromosomal DNA
- *M. tuberculosis* lacks resistance transposons
- Antibiotic resistance is the consequence of mutations in “housekeeping genes”
- Synonymous base pair changes are rare
- **Non-synonymous changes in drug resistance targets correlate with resistance TB lacks**
## Drug Resistance Target Genes

<table>
<thead>
<tr>
<th>Antituberculosis 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></td>
<td>rrs</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Rifampin</td>
<td>rpoB</td>
<td>β subunit of RNA polymerase</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>katG</td>
<td>Catalase-peroxidase</td>
</tr>
<tr>
<td></td>
<td>oxyR-ahpC</td>
<td>Alkylhydroreductase</td>
</tr>
<tr>
<td></td>
<td>inhA</td>
<td>Enoyl-ACP reductase</td>
</tr>
<tr>
<td></td>
<td>kasA</td>
<td>β-Ketoacyl-ACP synthase</td>
</tr>
<tr>
<td></td>
<td>ndh</td>
<td>NADH dehydrogenase</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>embCAB</td>
<td>Arabinosyltransferases</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>pncA</td>
<td>Amidase</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>inhA</td>
<td>Enoyl-ACP reductase</td>
</tr>
<tr>
<td></td>
<td>ethA</td>
<td>Flavoprotein monooxygenase</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>rrs</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Fluoroquinolone</td>
<td>gyrA</td>
<td>DNA gyrase α subunit</td>
</tr>
<tr>
<td></td>
<td>gyrB</td>
<td>DNA gyrase β subunit</td>
</tr>
<tr>
<td>Capreomycin</td>
<td>tlyA</td>
<td>rRNA methyltransferase</td>
</tr>
<tr>
<td></td>
<td>rrs</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Para-aminosalicylic acid</td>
<td>thyA</td>
<td>Thymidylate synthase</td>
</tr>
</tbody>
</table>
All molecular methods to genotype drug resistance in *M. tuberculosis* requires allelic discrimination at the single nucleotide level
Rifampin Resistance & *rpoB* Mutations

**WT: CTG AGC CAA TTC ATG GAC CAG AAC CCG CTG TCG GGG TTG ACC CAC AAG CGC CGA CTG TCG GCG CTG**

**RIF: CTG AGC CAA TTC ATG GAC CAG AAC CCG CTG TCG GGG TTG ACC TAC AAG CGC CGA CTG TCG GCG CTG**
Fluoroquinolone Resistance and *gyrA* Mutations

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<tr>
<th>Position</th>
<th>Wild Type</th>
<th>Resistance</th>
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<tr>
<td>74</td>
<td>GCC...CAC</td>
<td>GCC...CAC</td>
</tr>
<tr>
<td></td>
<td>Ala</td>
<td>Ala</td>
</tr>
<tr>
<td>88</td>
<td>GGC</td>
<td>GGC</td>
</tr>
<tr>
<td></td>
<td>His</td>
<td>His</td>
</tr>
<tr>
<td>90</td>
<td>CAC</td>
<td>GCG</td>
</tr>
<tr>
<td></td>
<td>His</td>
<td>Ser</td>
</tr>
<tr>
<td>91</td>
<td>GCG</td>
<td>TCG</td>
</tr>
<tr>
<td></td>
<td>Ala</td>
<td>Ile</td>
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<tr>
<td>94</td>
<td>ATC</td>
<td>TAC</td>
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<tr>
<td></td>
<td>Tyr</td>
<td>Asp</td>
</tr>
<tr>
<td>95</td>
<td>GAC</td>
<td>AGC</td>
</tr>
<tr>
<td></td>
<td>Ser</td>
<td>Ser</td>
</tr>
<tr>
<td></td>
<td>Leu</td>
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<tr>
<th>Amino Acid</th>
<th>Wild Type</th>
<th>Resistance</th>
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<tbody>
<tr>
<td></td>
<td>Ser</td>
<td>Val</td>
</tr>
<tr>
<td></td>
<td>10 (11%)</td>
<td>54 (62%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 (15%)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Wild Type</th>
<th>Resistance</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Gly</td>
<td>Gly</td>
</tr>
<tr>
<td></td>
<td>42 (48%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 (3%)</td>
</tr>
<tr>
<td></td>
<td>Ala</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 (1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>26 (30%)</td>
</tr>
<tr>
<td></td>
<td>Tyr</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asn</td>
<td></td>
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</tr>
</tbody>
</table>

Wild Type:  
CAC GGC CAC GCG TCG ATC TAC GAC AGC CTG

Resistance:  
CAC GGC CAC GCG TCG ATC TAC GGC AGC CTG
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* – Detects MDR
  - 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
Molecular Epidemiology
Molecular Epidemiology

- Hybrid field incorporating molecular biology, epidemiology and clinical medicine

- Track strains in populations to indicate/refute transmission (e.g., outbreaks)
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
Genotyping Targets to Discriminate *M. tuberculosis*

Barnes et al. NEJM – 2003;349:1149
IS6110 DNA Fingerprinting

- Standardized methodology
- Southern blot hybridization
- Pvull restriction digest
- Common right-side hybridization probe
- Common molecular weight standards
- Digitized patterns
- Pattern matching software
IS6110 DNA Fingerprint
THE EPIDEMIOLOGY OF TUBERCULOSIS IN SAN FRANCISCO
A Population-Based Study Using Conventional and Molecular Methods

Peter M. Small, M.D., Philip C. Hopewell, M.D., Samir P. Singh, B.S., Antonio Paz, M.D.,
Julie Parsonnet, M.D., Delaney C. Ruston, B.S., Gisela F. Schecter, M.D., M.P.H.,
Charles L. Daley, M.D., and Gary K. Schoolnik, M.D.
Recurrent tuberculosis
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

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>M. avium</th>
<th>H_{37}Rv</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td></td>
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<td>B</td>
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<tr>
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<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

 kb  |
--- |---|---|---|---|---|---|---|---|---|---|
 6.4 | 4.4 | 2.3 | 2.0 | 1.3 | 1.0 | 0.9 |
Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment

12/16 patients relapsed with exogenous strains
(one HIV+ and 15 HIV-)
270 Asian and 220 African elephants in captivity
Estimated TB infected: 12% Asian, 2% African
ALBUQUERQUE, N.M. (AP) - Irene was depressed. She ignored her paints and brushes; she stopped balancing tires on her head for fun; she was nauseated and ate dirt, and the only food that interested her was small cottonwood branches to control her upset stomach.

She lost 2,000 pounds.

But today the 5-ton Asian elephant at the Rio Grande Zoo is feeling much better, after recovering from the side effects of medications for tuberculosis.

"It's been a very gratifying, satisfactory effort to see an enormous, incredible animal like this, whose life was really threatened by this infection, do so well," said Dr. Gary Simpson, medical director of the state Infectious Diseases Bureau.
Case Study

- New Mexico, 2000
- 3 elephants abused and malnourished by small circus caravan – one died
- 2 surviving elephants, Irene (Asian) and Donna (African) brought to Albuquerque zoo in New Mexico
- Quarantined for 6 months
- Monthly trunk washes
Trunk Wash
Case Study

- Irene had multiple positive trunk washes
- She was treated for tuberculosis and Donna was prophylaxed with INH
- No elephant handlers PPD converted and they were routinely monitored

- So – how do you treat an elephant?
CAREFULLY!
Treatment

- INH, PZA and Enrofloxacin
- 18 months
- Major hurdle: adequate supply of drug
- Bigger hurdle: could not dose orally
THE EXAMINATION ROOM
IRENE – THE ILL PATIENT
Syringe is prepared
ADHERENCE TO COMBINATION THERAPY
ADHERENCE TO COMBINATION THERAPY
Long Reach
IRENE
–
THE ILL PATIENT

Rozi
19 y/o F
Born at zoo (daughter of Alice)

Alice
37 y/o F
Wild born
Being Tx'ed for TB

Irene
44 y/o F
Wild born
S/P Tx for TB, 2000

Daizy
2 y/o F
(daughter of Rozie)
Case Study

- 2011
- Irene is cured over 9 years – trunk washes remain negative
- However, a single colony was recovered from a trunk wash from Alice
- Alice shared the barn with Irene toward the last 6 months of her treatment
Isolated *M. tuberculosis* from Alice (2000)
- 3 different strains (1-5) isolated from trunk washes
- EH strain (2-4) repeatedly cultured

Isolated *M. tuberculosis* from Irene (2011)
- EH strain culture from trunk wash (6)
WHOLE GENOME SEQUENCING
Strategy

- Select 1 of the 3 cultures from Irene and the lone isolate from Alice and compare their genomes
- Illumina Genome Analyzer used to sequence the two genomes
- The genomes were compared to the H37Rv scaffold
- SNP differences between the two strains were identified
200 distinguishing SNPs among 32 genomes
Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection

Christopher B. Ford¹,¹¹, Philana Ling Lin²,¹¹, Michael Chase¹, Rupal R. Shah¹, Oleg lartchouk³, James Galagan⁴,⁵,⁶, Nilofar Mohaideen⁷, Thomas R. Ioerger⁸, James C. Sacchettini⁷, Marc Lipsitch¹,⁹, JoAnne L. Flynn¹⁰, and Sarah M. Fortune¹

Inferring patient to patient transmission of *Mycobacterium tuberculosis* from whole genome sequencing data

Josephine M Bryant¹, Anita C Schürch²,³,⁴, Henk van Deutekom⁵, Simon R Harris¹, Jessica L de Beer², Victor de Jager³,⁸, Kristin Kremer², Sacha A F T van Hijum³,⁶,⁷, Roland J Siezen³,⁶, Martien Borgdorff⁵,⁸, Stephen D Bentley¹, Julian Parkhill¹ and Dick van Soolingen²,⁹

Estimated rate of change: ~0.3 SNP per genome per year
Isolated \textit{M. tuberculosis} from Alice (2000)

- 3 different strains (1-5) isolated from trunk washes
- EH strain (2-4) repeatedly cultured

Isolated \textit{M. tuberculosis} from Irene (2011)

- EH strain culture from trunk wash (6)

Selected EH strain in lanes 4 and 6 for whole genome sequencing
Whole genome comparison of the two strains identified isolates 11 years apart:

- 3 SNP differences between the two genomes
  - 2 non-synonymous changes
  - 1 synonymous change

*M. tuberculosis* maintains a stable genome following repeated infections in strains from clustered outbreaks, in laboratory non-human primates and now in elephants.
This study described one of the first successful treatment of an elephant in captivity with tuberculosis. Irene has now been trunk wash negative for more than 12 years.

The epidemiology, trunk wash protocol, mycobacteriology with the genomic data provides strong evidence that *M. tuberculosis* was transmitted between two elephants in captivity.