DNA Fingerprinting of *M. tuberculosis*: Lessons from molecular epidemiology and comparative genomics

Barun Mathema, MPH
Research Epidemiologist
Public Health Research Institute

**Tuberculosis Fact Sheet**

- 2 billion people infected with *M. tuberculosis (M.tb)*
- 9 million new cases each year
- 2 million deaths each year
- HIV co-infection
- Rise of increasingly drug-resistant forms
- 85% of the mortality in developing countries

*Mycobacterium tuberculosis*
HIV and Multidrug Resistance

HIV and Tuberculosis
- Co-infection of *M. tb* and HIV a deadly-duet
- 11% co-infected (range from 1% to over 60%)
- Results in reactivation of tuberculosis or rapid progression to disease

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

Mycobacterium tuberculosis

- Lipid-rich Gram positive bacterium
- Intracellular pathogen (alveolar macrophages)
- Airborne transmission
- Slow-growing (~24 hr)
- Long latency period (esp. among the immunocompetent)

Mycobacterium tuberculosis complex

- *M. tuberculosis*
- *M. bovis*, BCG
- *M. africanum*
- *M. microti*
- *M. canettii*
- *M. pinnipedii*
- *M. caprae*

  Conserved 16S sequences

*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
- Considered a monomorphic species
- Synonymous base pair changes are rare
- *M. tuberculosis* "young" human pathogen

**Principal Genetic Groups**

![Evolution Tree](image)

**GEOGRAPHIC DISTRIBUTION OF GENETIC CLUSTERS**

![Map](image)
Phylogenetic studies of *M. tuberculosis* strains have suggested a clonal population structure, i.e. very little, at the DNA sequence level, that is different between strains. Up to 9 discrete branches or lineages have been proposed.

However, from molecular epidemiology (e.g. IS6110, spoligotyping, MIRU), we know that there exists enormous variation between *M. tuberculosis* strains.

Molecular Epidemiology

- Hybrid field incorporating molecular biology, epidemiology and clinical medicine
- Track strains in populations to indicate/refute transmission (e.g. outbreaks)
- Investigate whether diverse clinical strains differ in their clinical/epidemiological properties
- Associating clinical/epidemiologic characteristics with biological properties
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?

Genotyping data – uses for public health

- 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

Molecular Markers and Methods

Primary Genotyping Method
- **IS6110 (RFLP)** 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*

-Barnes et al. NEJM – 2003;349:1149
PHRI TB CENTER

• >26,000 M. tuberculosis isolates
• Diverse geography – MDR isolates
• All fingerprinted using IS6110
• 25% spoligotyped
• >3000 sSNP analysis
• Patient and strain database
• Archived fingerprint library

Selected topics where utility of M. tuberculosis strain diversity has been realized

Laboratory Contamination of M. tuberculosis Cultures
Patient JH: Image # 1

- JH - 51 y/o male
- 12-15-04 – referred to a local NJ health department clinic with symptoms of TB. A sputum sample was collected and was 4+ on smear for AFB and grew M.tb that was resistant to INH and SM
- 12-17-04 – DOT was 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 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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</thead>
<tbody>
<tr>
<td>12-16-04</td>
<td>4+</td>
<td>M.tb</td>
<td>Sputum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-17-04</td>
<td>&gt;10</td>
<td>M.tb</td>
<td>Sputum</td>
<td>S12-166-5</td>
<td>INH, SM</td>
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<tr>
<td>12-20-04</td>
<td>1-5</td>
<td>M.tb</td>
<td>Sputum</td>
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<tr>
<td>1-3-05</td>
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<td>M.tb</td>
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<tr>
<td>1-14-05</td>
<td>Neg.</td>
<td>M.tb</td>
<td>Sputum</td>
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</tr>
</tbody>
</table>

A View of Laboratory Cross-Contamination

- 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) – 12-17-04
- 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 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 and was admitted from 10-31-04 – 11-15-04 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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</thead>
<tbody>
<tr>
<td>11-3-04</td>
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<td>Sputum</td>
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<tr>
<td>11-9-04</td>
<td>+</td>
<td><em>M.tb</em></td>
<td>Sputum</td>
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<tr>
<td>11-11-04</td>
<td>+</td>
<td><em>M.tb</em></td>
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<tr>
<td>11-16-04</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 lung function when treatment was stopped
- 7-16-04 – he was seen in clinic for routine follow-up 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
### Patient PS - bacteriology

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<thead>
<tr>
<th>Date</th>
<th>Smear</th>
<th>Culture</th>
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<th>Sp#</th>
<th>Sensitivity</th>
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</thead>
<tbody>
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<td>Sputum</td>
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<tr>
<td>8-15-03</td>
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<td>9-12-03</td>
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<tr>
<td>1-9-04</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Sputum</td>
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<tr>
<td>7-16-04</td>
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<td>Neg.</td>
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<tr>
<td>12-17-04</td>
<td>Neg.</td>
<td>M.tb</td>
<td>Sputum</td>
<td>S12-0172-72</td>
<td></td>
</tr>
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### Summary

- The integration of molecular typing of *M. tuberculosis* with traditional TB control programs improves public health
- Cluster analysis helps to prioritize resources and provides a direct measurement of transmission
- The most significant clinical and public health role for genotyping *M. tuberculosis* is to rapidly identify cases of laboratory cross-contamination

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


Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment

12/16 patients relapsed with exogenous strains (one HIV+ and 15 HIV-).
HIV-1 and recurrence, relapse and reinfection of tuberculosis after cure: a cohort study in South African mineworkers
Sonnenberg et al. Lancet 2001;358:1687-93

Recurrence among HIV+ patients were 2.4 times (hazard ratio) compared to HIV-, due to the strong association with exogenous events (HR: ∼18)

- Elegant work by Canetti in the early 1970s showed evidence or exogenous re-infection by looking at drug resistance
- Why does natural infection not protect against a secondary insult?
- Does the immune response of the initial infected strain matter in protecting or not protecting against a secondary infection (i.e. is this a strain specific phenomenon)?
- Complicates rational vaccine design and development

Drugs resistance

Treating Tuberculosis

- Limited arsenal of antitubercular drugs
- Combination therapy to prevent resistance
- Acquired vs. primary resistance
- Drug susceptibility testing ~ 1 month
- Drug resistance targets identified
- Rapid genotyping of drug resistance possible
Combination Therapy

• Streptomycin failed as a single drug
• Combination therapy required
• Two to four drugs – six to nine months
• Never add a single agent to a failing regimen
• Adherence to therapy improved by DOTS

Acquired vs Primary Resistance

Acquired resistance
• Nonadherence to therapy
• Inappropriate therapy due to poor regimen selection, erratic drug supply

Primary resistance
• Nosocomial transmission
• Community transmission

Drug Susceptibility Testing

• *M. tuberculosis* slow growth confounds classical drug susceptibility testing
• One month turnaround time is common
• Second line drug testing to determine XDR only done on MDR strains

Antitubercular Antibiotics

<table>
<thead>
<tr>
<th>ANTIBIOTICS</th>
<th>YEAR</th>
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<tbody>
<tr>
<td>Streptomycin</td>
<td>1944</td>
</tr>
<tr>
<td>PAS</td>
<td>1949</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>1952</td>
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<tr>
<td>Pyrazinamide</td>
<td>1954</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>1955</td>
</tr>
<tr>
<td>Capreomycin</td>
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</tr>
<tr>
<td>Ethionamide</td>
<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>1962</td>
</tr>
<tr>
<td>Rifampin</td>
<td>1963</td>
</tr>
<tr>
<td>Kanamycin</td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolones</td>
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</table>
Genotyping Drug Resistance

- Nearly all drug resistance target genes identified
- With two exceptions, non-synonymous mutations in drug resistance target genes predicts resistance
- Molecular approaches are able to genotype resistance in less than 24 hrs – too costly even for developed countries

Drug Resistance Target Genes

<table>
<thead>
<tr>
<th>Antituberculosis agent</th>
<th>Gene</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td>myl</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>katG</td>
<td>Catalase-peroxidase</td>
</tr>
<tr>
<td></td>
<td>espC</td>
<td>Alkyl hydroperoxidase</td>
</tr>
<tr>
<td></td>
<td>inhA</td>
<td>Enoyl-ACP reductase</td>
</tr>
<tr>
<td></td>
<td>rpsL</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>embC</td>
<td>Ethambutol dehydrogenase</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>pncA</td>
<td>Amikacin</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>ethA</td>
<td>Ethionamide dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>ethC</td>
<td>Ethionamide monooxygenase</td>
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<tr>
<td>Kanamycin</td>
<td>kanR</td>
<td>16S rRNA</td>
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<tr>
<td>Fluoroquinolone</td>
<td>gnd</td>
<td>DNA gyrase α subunit</td>
</tr>
<tr>
<td></td>
<td>gndβ</td>
<td>DNA gyrase β subunit</td>
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<tr>
<td>Capreomycin</td>
<td>rhlA</td>
<td>β-hydroxy fatty acid chain oxidase</td>
</tr>
<tr>
<td></td>
<td>rhlA</td>
<td>β-hydroxy fatty acid chain oxidase</td>
</tr>
<tr>
<td>Para-aminosalicyclic acid</td>
<td>pah</td>
<td>Thymidylate synthase</td>
</tr>
</tbody>
</table>
The occurrence of multidrug resistant tuberculosis (MDR-TB) is a major public health concern as it is costly and complicates treatment yielding poor therapeutic indices.

MDR-TB may be cured by short-course chemotherapy in some, while in others bacillary growth is merely suppressed as longs as treatment is continued.

Between 8 and 35% of patients have persistently active disease refractory to multidrug therapy and cure rates, even among HIV-negative patients, remain well below that of drug susceptible patients.

To evaluate the natural history of MDR-TB in the human lung we sampled serial sputum isolates and studied the genetic characteristic and evolution of drug resistance at the genetic level.

- 13 HIV-negative MDR-TB patients from the Western Cape, South Africa who were refractory to at least 12 months of chemotherapy were studied.
- Isolates from serial sputum samples were collected over the 56 weeks study period (mean 7.6 – range 2-16 cultures/patient).
- IS6110-based RFLP, spoligotyping, and target sequencing of rpoB, katG, inhA, pncA, embB, rpsL, rrs and gyrA all were carried out using standardized protocols.

### Drug Resistance Target Genes

<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>Gene</th>
<th>Product</th>
</tr>
</thead>
<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>
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<td>katG</td>
<td>Catalase-peroxidase</td>
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<tr>
<td></td>
<td>embB</td>
<td>ATP-dependent deoxyuridylase</td>
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<td>inhA</td>
<td>Endo-ACP reductase</td>
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<td>rpsL</td>
<td>β-Ketoacyl-ACP synthase</td>
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<td>rrs</td>
<td>NADH dehydrogenase</td>
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<td>Ethambutol</td>
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<td>Pyrazinamide</td>
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<td>rrs</td>
<td>NADH dehydrogenase</td>
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<td>Kanamycin</td>
<td>rrl</td>
<td>Fluoroprotein monoxygenase</td>
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<td>rpsL</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Rifampin</td>
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<td>DNA gyrase subunit</td>
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<tr>
<td>Capreomycin'</td>
<td>rpsL</td>
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</tr>
<tr>
<td></td>
<td>rpsL</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>Para-aminosalicylic acid'</td>
<td>shd</td>
<td>Thymidylate synthase</td>
</tr>
</tbody>
</table>
• In all patients, chronic MDR-TB was caused by a single *M. tuberculosis* strain that did not vary by IS6110 fingerprint and spoligotype

• Isolates from 4/13 patients had acquired additional drug resistance mutations during treatment

• In these 4 patients, heterogenous populations containing a mixture drug susceptible/resistant bacilli with different mutations were observed

Genotypic polymorphism of selected *M. tuberculosis* genes

- Acquisition of new drug resistance (a stochastic event) during treatment of TB may result in mixtures of bacilli (R and S) in the lung as well as in any given sputum sample
- This suggests that drug resistance may arise independently in distinct microenvironments in the human lung – supported by studies of discrete lung lesions
- Genetic heterogeneity requires treatment targeted at both resistant and susceptible bacillary phenotypes
- Suggest that the founder strain may undergo differential evolutionary trajectories within discrete environments
When and where?
March 1992, 168th Street, NYC

Tuberculosis in New York City

Tuberculosis Cases and Rates New York City, 1982 – 2006

- Number of Cases
- Rate/100,000

11.9
22.4
55.0

82 84 86 88 90 92 94 96 98 00 02 04 06

954 Cases in 2006
Causes of Resurgent Tuberculosis in 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

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.
Munaff et al., JID 2003:188;356.

Multidrug Resistant Strain W

- Isoniazid (100%)
- Rifampin (100%)
- Streptomycin (100%)
- Ethambutol (100%)
- Pyrazinamide (85%)
- Kanamycin (92%)
- Fluoroquinolones (0%)

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>AGG; Lys>Arg
- Ethambutol (100%) embB - 306:ATG>GTG; Met>Val
- Pyrazinamide (55%) pncA - 139:ACC>GCC; Thr>Ala
- Kanamycin (92%) rrs - nucleotide 1400
- Fluoroquinolones (0%)
The New face of TB in South Africa: XDR and HIV

15 YEARS LATER...

Multidrug Resistance (MDR)
Resistance to at least isoniazid (INH) and rifampin (RIF)

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)

KwaZulu Natal, South Africa

- Jan 2005 – March 2006
- 1,539 TB Diagnosed
- 542 Culture Positive Cases
- 168 MDR
- 53 XDR
- 52/53 Died
- 44 Patients tested – All HIV+

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

Lancet 2006; 368: 1575-80

MEDIAN SURVIVAL OF 16 DAYS FROM THE TIME OF DIAGNOSIS
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

As we learned in NYC during our HIV – MDR outbreak, it will take strong political will, dedicated medical and public health teams and a great deal of money to deal with this emerging epidemic

• If not, XDR and all its baggage will appear at your doorsteps

Definitions

• Single nucleotide polymorphisms (SNPs): is a DNA sequence variation in a single nucleotide that can encode the same, synonymous (sSNPs), or different, nonsynonymous (nsSNPs), amino acid

• Phylogenetics: deals with the study of evolutionary relations among various species or populations of organisms, through molecular sequencing data

• Lineage: descent from a common progenitor

• Clone: a group of genetically identical cells descended from a single common ancestor, arising from a single original cell as a result of binary fission

• Genotype: is a group of organisms sharing a specific genetic constitution under consideration, e.g. number and location of insertion sequences such as IS6110