Diagnosis of Drug Resistant TB

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Hinduja Hospital
Diagnostics for tuberculosis: Time to usher in a new era

Lack of diagnostic capacity has been a crucial barrier

At least 20 new technologies in different stages of development

Expanded access to new diagnostics
Expanding lab capacity is a global priority

Strengthening TB labs from unimaginable …to indispensable
Requirements for Drug Susceptibility Tests

- High intra & inter lab reproducibility
- Shortest TAT
- Distinguish between high & low levels of R
- Practical & affordable
- Minimal investment & consummable costs
- Minimal labor time
- Applicability to 1\textsuperscript{st} & 2\textsuperscript{nd} line drugs
DST - Drug Susceptibility Testing

1. Growth observation

2. Detection of Metabolic activity or products

3. Phage based technologies

4. Molecular methods
### 1. Growth observation - Indirect methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Proportion method</strong></td>
<td>Ratio of colonies on the drug medium to those on drug-free medium (set at 1%)</td>
</tr>
<tr>
<td></td>
<td>If &gt;1% is R, the drug will not be useful in therapy</td>
</tr>
<tr>
<td></td>
<td>Qualitative as well as precise estimate</td>
</tr>
<tr>
<td><strong>2. Absolute concentration method or MIC</strong></td>
<td>Growth is taken as the end point (&gt;20 colonies)</td>
</tr>
<tr>
<td></td>
<td>Affected by inoculum size &amp; viability</td>
</tr>
<tr>
<td><strong>3. Resistance ratio method</strong></td>
<td>Determines the RR between the MIC of the test strain &amp; MIC of reference strain</td>
</tr>
<tr>
<td></td>
<td>( RR &gt;8)</td>
</tr>
<tr>
<td></td>
<td>Affected by variations in std strains</td>
</tr>
</tbody>
</table>
Problems in DST

- Inoculum standardisation (number / dispersion / viability)
- Stability of drugs (CLSI considers LJ to be unsuitable)
- Alteration of drugs in different media (inactivation / protein binding / deterioration / inspissation / pH / antagonistic substances / incomplete dissolution in solvents / inaccurate dilution)
- Incubation temp / time
- Criteria of resistance
- Type of test performed
Drug Susceptibility Testing (DST)

1. Growth observation

   **Rapid Assays : Directly from clinical specimens**
   - Nitrate Reduction Assay - NRA
   - Microscopic Observation Drug Sus – MODS
   - Thin Layer Agar - TLA

2. Detection of Metabolic activity or products

3. Newer methods as Phage based technologies

4. Molecular methods : detection of genetic mutations
Direct assays: Rapid screening for MDR

1. Nitrate Reductase Assay (Greiss)
   Color change > than the Control

Pooled sensitivities for detection of INH & Rif R 94 & 96%

*BMC Infect Dis 2009;9:67*
Direct assays for resource limited settings
accurate case detection & simultaneous identification of MDR

2. Microscopic Observation Drug Susceptibility Assay – MODS

Characteristic tangles of *M. tuberculosis* seen under inverted microscope (1-3 weeks)
Pooled sensitivities for detection of INH & Rif R 92 & 96 %

*BMC Infect Dis* 2009;9:67
Direct assays for resource limited settings

3. Thin Layer Agar culture – TLA 7H10 (.MODS for solid media)

Alternate day microscopy

Sensitivity, specificity & predictive values for I & R 100%

1. Growth observation

2. Detection of Metabolic activity or products
   MGIT / MB Bact / ESP Myco
   DST: Time to detection is 10-14 days as opposed to 3 - 4 weeks with agar

3. Phage based technologies

4. Molecular methods
Critical Concentrations

- Need for unanimous agreed standards to secure reproducibility of DST of 2\textsuperscript{nd} line drugs

- For 2\textsuperscript{nd} line drugs 21 SRL had different Critical Conc due to variation in testing systems & methods

- QA for 2\textsuperscript{nd} line drugs

*Int J Tubercle Lung Dis* 2004;8:1157
Drug susceptibility testing of *Mycobacterium tuberculosis* against second-line drugs using the Bactec MGIT 960 System

C. Rodrigues,* J. Jani,† S. Shenai,* P. Thakkar,* S. Siddiqi,** A. Mehta*

* Department of Microbiology, P D Hinduja National Hospital and Medical Research Centre Tertiary Care Hospital, Mumbai, †Becton Dickinson Diagnostic Systems, Becton Dickinson, Mumbai, India; **Becton Dickinson Diagnostic Systems, Sparks, Maryland, USA

Detects O$_2$ consumption in the presence or absence of drug

2. Detection of Metabolic activity or products

Colorimetric Redox Indicators: CRI assays (8-12 days)

MTT - Methyl Thiazol diphenyl Tetrazolium bromide
MABA - Microplate Alamar Blue Assay
REMA - REsazurin Microtitre Assay

Rapid, low cost
show good agreement
for H & R
standard for E & Z
? Biosafety & contam
Drug Susceptibility Tests (DST) – *M. tuberculosis*

1. Growth observation

2. Detection of Metabolic activity or products

3. Phage based technology: LRP, PhaB, Fast Plaque

4. Molecular methods: detection of genetic mutations
Lysis with mycobacteriophages

Rifampicin Sensitive

Plate without Rifampicin

Plate with Rifampicin

Rifampicin Resistant

Plate with Rifampicin

Plate without Rifampicin
## Phage assay

<table>
<thead>
<tr>
<th>Test</th>
<th>Ref. Std</th>
<th>Specimen</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIF Resistance</td>
<td>BACTEC 460</td>
<td>Culture Isolates</td>
<td>Sensitivity 96%</td>
</tr>
<tr>
<td></td>
<td>L.J Proportion mtd</td>
<td></td>
<td>Specificity 100%</td>
</tr>
<tr>
<td>INH Resistance</td>
<td>BACTEC 460</td>
<td>Culture Isolates</td>
<td>Sensitivity 97%</td>
</tr>
<tr>
<td></td>
<td>L.J Proportion mtd</td>
<td></td>
<td>Specificity 100%</td>
</tr>
<tr>
<td>RIF Resistant</td>
<td>BACTEC 460</td>
<td>Smear +</td>
<td>Sensitivity 93%</td>
</tr>
<tr>
<td></td>
<td>Resp. Spec</td>
<td></td>
<td>Specificity 87%</td>
</tr>
</tbody>
</table>

**Limitations**

- Analytical sensitivity: 100-300 bacilli/ml
- Not useful in smear negative, paucibacillary specimens
- Pts receiving anti-TB treatment

*Ajay K et al. IJMM 2002;20:211-14*
Drug Susceptibility Testing (DST): *M. tuberculosis*

1. Growth observation

2. Detection of Metabolic activity or products

3. Phage based technology

4. Molecular methods: detection of genetic mutations
   advances in genotypic hardware
There is no doubt that the gene is by far the most sophisticated program around........

Bill Gates
<table>
<thead>
<tr>
<th>Drug</th>
<th>Gene</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>RNA polymerase subunit B (rpo(\beta))</td>
<td>96%</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Enoyl acp reductase ((inhA))</td>
<td>10-20%</td>
</tr>
<tr>
<td></td>
<td>Catalase–peroxidase ((katG))</td>
<td>30-60%</td>
</tr>
<tr>
<td></td>
<td>Alkyl hydroxyperoxideoxy reductase ((ahpC))</td>
<td>2-8%</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Arabinosyl transferase ((embC,A,B))</td>
<td>80%</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Ribosomal protein subunit 12 ((rpsL))</td>
<td>52-59%</td>
</tr>
<tr>
<td></td>
<td>16 S ribosomal RNA ((rrs))</td>
<td>8-21%</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>Pyrazinamidase-nicotinamidase ((pncA))</td>
<td>72-97%</td>
</tr>
<tr>
<td>Quinolones</td>
<td>DNA gyrase subunit A/B ((gyr A/B))</td>
<td>65-90%</td>
</tr>
</tbody>
</table>
Limitations of Culture Methods

- Long turn around time (weeks to months)
- Often fail precise species identification
- Cultures maybe negative for patients on treatment
- Laborious susceptibility testing
  - standardization of critical concentrations
  - establish an appropriate inoculum size
  - stability of drug in different culture media
  - reliability of results
  - lack of QA
Molecular Methods to diagnosis drug resistance

“Leap frogging technology” - advantages

- Rapid
- Sensitive
- Good performance characteristics
- Direct application to clinical specimens
- Less biohazard risk
- Feasibility of automation & high throughput
- Resistance genomically based
Molecular Methods to diagnosis drug resistance
“Leap frogging technology” - limitations

- Poly resistance or multiple genes involved
  (Resistance may involve several distinct loci)
- Infrastructure / experienced staff / cost
- Risk of false positive results
- Target only known or described mutations
- Silent mutations
- Dual infections
Drug susceptibility Tests - Molecular

- DNA Sequencing
- PCR SSCP
- Solid phase hybridization assays
- Real time formats
- Microarrays
DNA Sequencing

- Reference standard for mutation detection
- Performed with automated sequencer
- Accurate & reliable
- Labor intensive & expensive, cannot be routinely performed

Wild Type

Mutant

katG gene

S315T
Pyrosequencing - Chemistry

- Chemistries
- ATP
- Light
- Signal
Detecting a $rpo\beta$ mutation

Complementary nucleotide to the base strand is accompanied by release of pyrophosphate (PPi) which generates light.
DST : PCR - SSCP

q Majority of RifR isolates had mutations at codon S531L[TCG-TTG] (66%) followed by H526D [CAC-GAC] (20%)

q 80% of INH R strains had mutations at katG gene codon S315T[AGC-ACC]

High Incidence of the Beijing Genotype among Multidrug-Resistant Isolates of Mycobacterium tuberculosis in a Tertiary Care Center in Mumbai, India

Deepak Almeida, Camilla Rodrigues, Tester F. Ashavaid, Ajit Lalvani, Zarir F. Udwadia, and Ajita Mehta

1Research Labs, and Departments of 2Microbiology, 3Biochemistry, in the literature [14–19], and there is only 1 study from Mumbai [20]. We therefore performed fingerprinting analysis of our drug-susceptible and multidrug-resistant isolates to determine whether there is any significant clonal spread in our geographic region. Also, fingerprinting analysis would serve to answer the following questions: (1) Does the Beijing genotype contribute significantly to the burden of tuberculosis in India? (2) If so, is the Beijing genotype preferentially associated with multidrug resistance?

Methods. Our hospital is a tertiary care center with a referral bias toward nonresponding cases; it is located in central
Drug susceptibility Tests - Molecular

- Solid phase hybridization assays
  - Line Probe Assays : LPA
Hain GenoType MTBDRplus
from evidence to policy

- Strip 1: MDR TB
- Strips 2+3: WT
- Strip 4: MDRTB
- Strip 5->7: WT
- Strip 8: INH resistance
- Strips 9->12: WT

S + Pooled sensitivities & specificities : 98.4 & 98.9%

Eur Respir J 2008 ;32:1165
Hain Genotype MTBDR s/
detecting resistance to fluoroquinolones & aminoglycosides
Amplified PCR products of resistant genes are hybridized to labeled probes complementary to wild or mutant sequences.

These are visualized by arrays autoradiography, enhanced chemiluminescence, alkaline phosphatase, gel drops by fluorescence.
Rapid speciation of 15 clinically relevant mycobacteria with simultaneous detection of resistance to rifampin, isoniazid, and streptomycin in *Mycobacterium tuberculosis* complex

Shubhada Shenai, Camilla Rodrigues *, Ajita Mehta

Int J Infect Dis 2009;13:46-58
Probes
1. MYC
2. MTB
3. Rif wt 1
4. Rif wt 2
5. Rif wt 3
6. Rif wt 4
7. Rif wt 5
8. Rif 533 mt
9. Rif 531 TTG
10. Rif 531 TGG
11. Rif 526 TAC
12. Rif 526 GAC
13. Rif 526 CGC
14. Rif 526 CTC
15. Rif 526 TGC
16. Rif 526 AAC
17. Rif 522 TTG
18. Rif 522 TGG
19. Del codon 516
20. Rif 516 TAC
21. Rif 516 GTC
22. Rif 516 GTG
23. Rif 516 GGC
24. Rif 513 CGC
25. Rif 511 CCA
26. Inh A wt
27. Inh A mt
28. KG 315 wt
29. KG 315 mt
30. KG 463 wt
31. KG 463 P
32. RpsL 43 wt
33. RpsL 43 mt
34. RpsL 88 wt
35. RpsL 88 mt
36. RRS 491 wt
37. RRS 491 mt
38. RRS 513 wt
39. RRS 513 mt
40. RRS 516 mt

Blot for MTB Drug R Testing

Wild – Susceptible, Mutant - Resistant
Increasing incidence of fluoroquinolone-resistant *Mycobacterium tuberculosis* in Mumbai, India

D. Agrawal,* Z. F. Udwadia,* C. Rodriguez,† A. Mehta*

*Department of Respiratory Medicine, and †Department of Microbiology, P D Hinduja National Hospital and Medical Research Centre, Mumbai, India

Fluoroquinolone resistance: QRDR in *gyrase A*

<table>
<thead>
<tr>
<th>74</th>
<th>88</th>
<th>90</th>
<th>91</th>
<th>94</th>
<th>95</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCC</td>
<td>CAC</td>
<td>GCC</td>
<td>CAC</td>
<td>GCC</td>
<td>TCG</td>
</tr>
<tr>
<td>Ala</td>
<td>His</td>
<td>Gly</td>
<td>His</td>
<td>Ala</td>
<td>Ser</td>
</tr>
</tbody>
</table>

- TCC
- GGC
- ACC
- Ser (40%)
- Thr (23%)

- GTG
- CCG
- GCC
- Pro (10%)
- AAC
- Asn (8%)
- TAC
- Tyr

(18.3%)

Fluoroquinolone resistance: QRDR in *gyrase A*
## Table: Drug Resistance Mutations

<table>
<thead>
<tr>
<th>No.(%) of isolates</th>
<th>Gene</th>
<th>Drug</th>
<th>Mutation seen</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 (100%)</td>
<td>kat G</td>
<td>Isoniazid</td>
<td>G 315C</td>
</tr>
<tr>
<td>21 (14%)</td>
<td>inhA</td>
<td>Isoniazid</td>
<td>C -15 T</td>
</tr>
<tr>
<td>146 (97%)</td>
<td>rpo B</td>
<td>Rifampicin</td>
<td>C 531T</td>
</tr>
<tr>
<td>4 (3%)</td>
<td>rpo B</td>
<td>Rifampicin</td>
<td>A 526 G</td>
</tr>
<tr>
<td>37 (25%)</td>
<td>gyrA</td>
<td>Fluoroquinolones</td>
<td>C 90 T</td>
</tr>
<tr>
<td>2 (1%)</td>
<td>gyrA</td>
<td>Fluoroquinolones</td>
<td>T 91C</td>
</tr>
<tr>
<td>79 (53%)</td>
<td>gyrA</td>
<td>Fluoroquinolones</td>
<td>A 94 G</td>
</tr>
<tr>
<td>18 (12%)</td>
<td>gyrA</td>
<td>Fluoroquinolones</td>
<td>A 94 C</td>
</tr>
<tr>
<td>14 (9%)</td>
<td>gyrA</td>
<td>Fluoroquinolones</td>
<td>G 94 A</td>
</tr>
<tr>
<td>106 (71%)</td>
<td>rrs</td>
<td>Kana/ amik / capreo</td>
<td>A 1401G</td>
</tr>
<tr>
<td>42 (28%)</td>
<td>rrs</td>
<td>Kana/ amik/capreo</td>
<td>G 1484 T</td>
</tr>
<tr>
<td>13 (31%)</td>
<td>tylA,rrs</td>
<td>Capreomycin</td>
<td>A205G</td>
</tr>
</tbody>
</table>
XDR blot to detect all known mutations in Isoniazid, Rifampicin, Kanamycin, Amikacin, Capreomycin, Ofloxacin, Moxifloxacin

MYC, MTB,
Rif 1 WT 509-514
Rif 2 WT 514-520
Rif 3 WT 521-525
Rif 4 WT 524-529
Rif 5 WT 530-534
Rif 533 CCG
Rif 531 TTG
Rif 531 TGG
Rif 526 TAC
Rif 526 GAC
Rif 526 TGC
Rif 526 AAC
Rif 526 CGC
Rif 516 del
Rif 522 TTG
Rif 522 TGG
Rif 516 TAC
Rif 516 GTC
Rif 516 GTG
Rif 516 GGC
Rif 513 CCA
InhA wt
Inh A mt
kat G wt
katG mt
gyA90 wt
gyA90 mt
gyA91 mt
gyA94 wt
gyA94 mt1
gyA94 mt2
gyA94mt3
gyA94 mt4
gyA95mt
gyB wt
gyB mt
rrs 1401 wt
rrs 1401 mt
rrs 1402 mt
rrs 1484 wt
rrs 1484 mt
<table>
<thead>
<tr>
<th></th>
<th>Identification Rate</th>
<th>Identification Time</th>
<th>Susceptibility testing Efficiency</th>
<th>Susceptibility testing Time</th>
<th>Total Time for Identification &amp; Susceptibility testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.J</td>
<td>30-50%</td>
<td>2-6 wks</td>
<td>80-99%</td>
<td>2-4 wks</td>
<td>4-12 wks</td>
</tr>
<tr>
<td>BACTEC</td>
<td>50-75%</td>
<td>2-3 wks</td>
<td>92-100%</td>
<td>8-12 days</td>
<td>3-5 wks</td>
</tr>
<tr>
<td>MGIT</td>
<td>50-75%</td>
<td>1-2 wks</td>
<td>98.6-100%</td>
<td>6-10 days</td>
<td>2-4 wks</td>
</tr>
<tr>
<td>RLBH</td>
<td>95-98%</td>
<td>2 days</td>
<td>98-100%</td>
<td>2 days</td>
<td>2 days</td>
</tr>
</tbody>
</table>

Technology transfer to X Cyton, Bangalore
## Ideal test for TB

- Rapid
- Directly from the specimen with sensitivity of culture
- Simultaneous detection of resistance
- On demand availability
- Single patient testing
- Easy
- Reproducible
- Robust

*Oh and should be really *really* cheap!*
Drug susceptibility Tests

- DNA Sequencing
- PCR SSCP
- Solid phase hybridization assays
- Real time formats
- Microarrays / chips
Molecular beacons for established mutations

Based on stem & loop structure with the probe in the loop
Fluorescence detected in Real time within 4 hrs w/o post PCR manipulation

J Clin Microbiol 2004;42 :4204
Integration of technologies

- Molecular beacons
- RT PCR
- Resistance-associated mutations
- Fluorimetric probes
- Microfluidics
- Sonic bacterial lysis
The assay is based on multiplex, nested real-time PCR with molecular beacons to detect MTB & to diagnose RIF resistance.
Rapid Molecular Detection of Tuberculosis and Rifampin Resistance

Catharina C. Boehme, M.D., Pamela Nabeta, M.D., Doris Hilleman, Ph.D., Mark P. Nicol, Ph.D., Shubhada Shenai, Ph.D., Fiorella Krapp, M.D., Jenny Allen, B.Tech., Rasim Tahirli, M.D., Robert Blakemore, B.S., Roxana Rustomjee, M.D., Ph.D., Ana Milovic, M.S., Martin Jones, Ph.D., Sean M. O’Brien, Ph.D., David H. Persing, M.D., Ph.D., Sabine Ruesch-Gerdes, M.D., Eduardo Gotuzzo, M.D., Camilla Rodrigues, M.D., David Alland, M.D., and Mark D. Perkins, M.D.
<table>
<thead>
<tr>
<th>Site</th>
<th>n</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All Culture-Positive</td>
<td>Smear-Positive and Culture-Positive</td>
</tr>
<tr>
<td>Lima, Peru</td>
<td>211</td>
<td>(99.1) 96.6–99.7</td>
<td>100 (98.1–100.0)</td>
</tr>
<tr>
<td>Baku, Azerbaijan</td>
<td>149</td>
<td>96.6 (92.4–98.6)</td>
<td>100 (95.4–100.0)</td>
</tr>
<tr>
<td>Cape Town, South Africa</td>
<td>148</td>
<td>95.9 (91.4–98.1)</td>
<td>99 (94.3–99.8)</td>
</tr>
<tr>
<td>Durban, South Africa</td>
<td>45</td>
<td>95.6 (85.2–98.8)</td>
<td>100 (88.6–100.0)</td>
</tr>
<tr>
<td>Mumbai, India</td>
<td>188</td>
<td>98.4 (95.4–99.5)</td>
<td>100 (99.7–100)</td>
</tr>
</tbody>
</table>

### Sensitivity & Specificity of Xpert TB/RIFas per test no. / patient

<table>
<thead>
<tr>
<th>No. of MTB/ RIF tests</th>
<th>n</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All Culture-Positive</td>
<td>Smear-Positive and Culture-Positive</td>
</tr>
<tr>
<td>3 Samples</td>
<td>741</td>
<td>97.6 (96.2–98.5)</td>
<td>99.8 (99.0–100.0)</td>
</tr>
<tr>
<td>(2 pellet and 1 direct)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Samples</td>
<td>1482</td>
<td>96 (94.6–97.1)</td>
<td>99.4 (98.6–99.7)</td>
</tr>
<tr>
<td>(1 pellet and 1 direct)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Sample</td>
<td>732</td>
<td>92.2 (90.0–93.9)</td>
<td>98.2 (96.8–99.0)</td>
</tr>
<tr>
<td>(direct)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Xpert MTB/RIF in a high HIV prevalence setting

HIV co infection may impact assay performance
S-C+ 55%
Outperformed Smear,
ruled in TB 18% relative increase in case detection
& accurately ruled out MDR

AJRCCM 2011 in press
Advantages

- Closed, self-contained, fully-integrated & automated platform
- Combines TB detection with Rif resistance detection
- Results within 2 hours
- Sensitivity almost ≈ culture
- Minimal hands on technical time
- Low safety level lab required
Disadvantages

- Cost for machine and consumables
- Relatively low PPV value in low MDR prevalence settings
We need a roadmap ahead now....
Evidence does not make decisions, people do....

Bryan Haynes
Current gaps

• Reduce time to detection

• Determine agreement rapid tests & standard DST

• Genetic basis of discordant results (new regions /points of difference )

• Cost effectiveness (private sector)

• PV of resistance associated mutations in determining sputum conversion
Unmet needs - “all TB patients matter”

- Define priorities

- National lab policy with a tiered network

- Work towards universal access & building capacity

- No single stand alone test, need to leverage molecular methods with culture

- Validation & QA/QC
Some clinical challenges…..

• Simultaneous infections with different strains (17% in new & 23% in previously treated) leading to conflicting DSTs

? Detection limit of molecular tests when testing heteroresistant populations (wild type & mutant)

• In culture negative pts on treatment, is it paradoxical (IRIS) or MDR TB or both?
Thank you