Phenotypic and Molecular Tests for Diagnosis and Drug Susceptibility Testing

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Bacteriological diagnosis of TB

1. Direct smear
2. Molecular resistance detection
3. Culture
4. Phenotypic drug susceptibility testing
Microscopy of stained smears

1. Auramine
2. (cold) Ziehl-Neelsen

both based upon acido-alcohol resistance
Auramine staining

Auramine - acid/alcohol – red thiazone

Auramine Positive Acid Fast Bacilli

Fluorescent microscopy
20X
40X

- suggests mycobacteria
- fast
- not specific → ZN
(cold) Ziehl-Neelsen staining
(on the same slide)
Fushine - Acid/alcohol - Methylene blue

ZN positive
Acid Fast Bacilli

Optical immersion microscopy X 100
A.F.B. positive

Ziehl-Neelsen Coloration Optical Microscope x100

suggests tuberculosis but could be non-tuberculous bacteria actinomycetes, rodococcus …
Direct Smear Quantification

WHO/The Union Codification

- **Suspect**: 1-2 AFB in > 200 fields (Repeat DS)
- **< 10**: 1 – 9 AFB in 100 fields
- **+**: 10 – 99 AFB in 100 fields
- **++**: 1 – 9 AFB / field
- **+++**: > 10 AFB / field

Estimation of the density
Direct Smear

• Fast, cheap, can be repeated on 3 specimens
• Poor sensitivity: \( > 5 \times 10^3 \) bacilli / mL
• Negative AFB does not eliminate TB
• Is not specific
• Does not evaluate viability
• If pulmonary TB, patient is contagious \( \rightarrow \) notification
Biological Diagnosis of TB

Is it TB?

Or non tuberculous mycobacteria

→ Mtb complex detection using molecular tests
Biological Diagnosis of TB

Is it TB?

Is it an antibiotic resistant TB?

Resistance is increasing and
80% MDR cases are not diagnosed or treated
Drug Resistant Tuberculosis

• **MDR-TB** = Isoniazid-resistant and rifampicin-resistant

• **XDR-TB** = MDR
  + Fluoroquinolone-resistant
  + 1 second line injectable drug-resistant
    (amikacin, kanamycin or capreomycin)
MDR molecular detection in clinical samples

*WHO endorsed assays*

- **Rifampicin resistance** \textit{rpoB}
  
  Xpert\textsuperscript{®} (Cepheid) MTB/RIF
  
  LPAs: GenoType\textsuperscript{®} (Hain) MTBDR\textit{plus}

  
  \textit{95\% of RIF}^R \textit{are also INH}^R

  \textit{and therefore MDR-TB}

- **Isoniazid resistance** \textit{katG, inhA}
  
  LPAs: GenoType\textsuperscript{®} (Hain) MTBDR\textit{plus}
Xpert® MTB/RIF (Cepheid)

Real Time PCR

*M. tuberculosis* complex DNA and Rifampicin resistance detection
Xpert® MTB/RIF

81 bp \( rpoB \) gene
Rifampicin resistance coding region

Hemi-nested PCR
5 probes bind to \( wt \) sequence
+ 1 amplification control probe
Results window

DNA detection

*M. tuberculosis* complex

No mutation associated with rifampicin resistance
Results window

DNA detection
M. tuberculosis complex (small quantity)

Rifampicin Resistance associated mutation
Results window

Assay Name: MTB Beta 8 for LC review-detect
Version: NA

<table>
<thead>
<tr>
<th>Analyte Name</th>
<th>Ct</th>
<th>EndPt</th>
<th>Analyte Result</th>
<th>Probe Check Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe D</td>
<td>0.0</td>
<td>2.0</td>
<td>NEG</td>
<td>PASS</td>
</tr>
<tr>
<td>Probe C</td>
<td>0.0</td>
<td>2.0</td>
<td>NEG</td>
<td>PASS</td>
</tr>
<tr>
<td>Probe E</td>
<td>0.0</td>
<td>-2.0</td>
<td>NEG</td>
<td>PASS</td>
</tr>
<tr>
<td>Probe B</td>
<td>0.0</td>
<td>6.0</td>
<td>NEG</td>
<td>PASS</td>
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<tr>
<td>Bg</td>
<td>27.4</td>
<td>260.0</td>
<td>PASS</td>
<td>PASS</td>
</tr>
<tr>
<td>Probe A</td>
<td>0.0</td>
<td>2.0</td>
<td>NEG</td>
<td>PASS</td>
</tr>
</tbody>
</table>

MTB not detected
Xpert® MTB/RIF

*M. tuberculosis* detection in pulmonary specimens

131 bacilli per mL sputum

Sensitivity: AFB+ 98%

AFB- averaging 70%

Specificity: 98.3%

Rifampicin resistance detection

Sensitivity 96.7%

Specificity 98.6%
GeneXpert® MTB/RIF

Advantages

- Rapid
- Safe, easy to use closed cartridge
- Detection Mtb complex and RIF resistance
- High sensitivity/specificity
GeneXpert® MTB/RIF

Limitations

- Cartridge’s shelf life
- Electricity, temperature, dust …
GeneXpert® MTB/RIF

Cepheid’s OMNI

- More rugged
- Battery
- Withstand dust and heat
- Fewer training requirement
GeneXpert® MTB/RIF

Molecular limitations

• Decreased capacity to detect \( rpoB \) C533G mutations
• Occasional false-positive RIF-resistance
  ➢ paucibacillary samples
  ➢ \( rpoB \) silent mutations (Q513Q, F514F)
GeneXpert® MTB/RIF ULTRA

- Larger chamber for DNA amplification
- 2 additional targets to detect TB (IS6110 and IS1081)
- Melting curve technology

→ Increased sensitivity only for bacilli detection
   We expect better sensitivity for children, HIV, extra-pulmonary specimens
→ The limit could be a reduced specificity
MDR detection

Antibiotic Resistance sequencing

Major target genes:

- $rpoB$  Rifampicin
- $katG$  Isoniazid
- $inhA$  Isoniazid

LIPAs

*GenoType® MTBDRplus* (Hain Lifescience)
Do the patient have MDR TB?

HAIN GenoType® MTBDRplus
Line Probe Assay
Mutations associated with RMP resistance

% of the indicated mutations in Rif-R strains (from Musser, 1995)
MDR detection

HAIN GenoType® MTBDRplus to confirm RIF-R
MDR detection?

HAIN GenoType® MTBDRplus

Rifampicin resistance is confirmed
MDR detection

HAIN GenoType® MTBDRplus to detect INH-R

Isoniazid

(inactive)

(catalase-peroxidase) KatG

NADH

Isonicotinic-acyl-NADH

InhA (enoyl ACP reductase)
(mycolic acid synthesis)
MDR detection

HAIN GenoType® MTBDR<sup>plus</sup> to detect INH-R

\[ RIF^R \quad INH^R \]

Isoniazid

(inactive)

(catalase-peroxidase)KatG

\[ \text{NADH} \]

Isonicotinic-acetyl-NADH

\[ \text{InhA} \quad \text{(enoyl ACP reductase)} \]

\[ \text{(mycolic acid synthesis)} \]

\[ = \text{MDR - TB} \]
GenoType® MTBDRplus

RMP and INH resistance detection compared to culture and clinical data

<table>
<thead>
<tr>
<th>Drug and smear status(^a)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pos</td>
<td>98.1</td>
<td>96.0</td>
<td>98.1</td>
<td>96.0</td>
</tr>
<tr>
<td>Neg</td>
<td>90.7</td>
<td>96.0</td>
<td>98.0</td>
<td>82.7</td>
</tr>
<tr>
<td>INH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pos</td>
<td>89.3</td>
<td>94.7</td>
<td>98.2</td>
<td>94.7</td>
</tr>
<tr>
<td>Neg</td>
<td>93.5</td>
<td>82.3</td>
<td>95.1</td>
<td>77.7</td>
</tr>
</tbody>
</table>

\(^a\) Pos, positive; Neg, negative.
The patient has **MDR TB**

*Can we give MDR treatment?*

*What about second line drugs susceptibility?*

*Does the patient have **XDR TB**?*
Molecular XDR detection

Major targets genes for antibiotic resistance sequencing:

- $gyrA$, $gyrB$ Fluoroquinolones
- $rrs$, $eis$ Amikacin/kanamycin/capreomycin
- $pncA$ Pyrazinamide

Line Probe Assays

- GenoType® MTBDRsl (Hain Lifescience)
Does the patient have XDR TB?

MDR patient A

HAIN GenoType® MTBDRsl

- **gyrA** → Fluoroquinolones
  - FQs

- **gyrB** → Fluoroquinolones

- **rrs** → KAN/AMK/CAP/VIO

- **eis** → KAN
  - KAN/AMK/CAPs

**Patient A does not have XDR TB**

9 months MDR treatment
Does patient B have XDR TB?

MDR patient A

MDR patient B

HAIN GenoType® MTBDRsl

Patient B has XDR TB

Treatment?

FQR (D94G)

KAN/AMK/CAP

MDR patient A

MDR patient B

HAIN GenoType® MTBDRsl

Patient B has XDR TB

Treatment?
Next molecular diagnosis assays

Xpert SL® XDR (Cepheid)

- Isoniazid (*katG, inhA* promoter)
- Fluoroquinolones (*gyrA, gyrB*)
- Amikacin, Kanamycin (*rrs, eis* promoter)

NGS (Illumina, Genoscreen)
Limitations of molecular tests

- cannot distinguish live bacilli
- Extracted DNA (quality, quantity)
- Outside target zone mutations
- Mutations in sensitive isolates
- Other resistance mechanism

→ *Culture*
→ *Phenotypic drug susceptibility testing*
Culture

- Viability of the mycobacteria
- Molecular tests
- Phenotypic drug susceptibility testing
Culture in L3

- Negative pressure
- Double door entrance
- Safety hood
- Protective mask

secure
expensive
Work with a *protective mask* (*FFP2*)
Samples Decontamination
Classic Petroff method

NaOH 4% - NALC
Centrifugation
Neutralization
Pellet resuspending buffer

NAC-PAC®AlphaTec
Direct smear
Inoculation

MGIT

LJ
Solid Medium Culture

- Löwenstein-Jensen or Coletsos
- Long time to positivity: 21 days
- Cultures reading 3 months
LJ medium at 37°C
Liquid Medium Culture

MGIT 960

• Liquid medium 7 mL
• Nutritional Supplement
• Antibiotics
  (Polymixine B, Azlocyline, Nalidixic acid, Trimetroprime, Amphothericin B)
Liquid Medium Culture

MGIT 960

- Incubate at 37°C
- During 56 days
- Consumption of oxygen detection
- Automatic reading every hour
Kudoh method

Sodium hydroxide, 4%
Ogawa modified medium pH 6.4

No centrifugation
Positive culture

Löwenstein Jensen Medium

Ogawa (Kudoh method)

*M. tuberculosis*

Rough colonies in 21 days
Solid Medium Culture

• Allows colony counts
• The aspect of colonies and the speed of growth gives clues as to the identification
• Several weeks delay …
Liquid Medium Culture MGIT

Faster, more sensitive

BD Bactec™ MGIT™ 960
Positive culture identification

Is it TB? **TB complex detection**

- Previous biochemical tests (niacine, nitrate reductase, catalase)
- Previous specific molecular probes (Geneprobe)
- **Rapid Immunochromatographic Assay**
  - (ex. SD Bioline’s TB Ag MPT64 Rapid Test)
- **Molecular tests:** GeneXpert® MTB/RIF or Line Probe Assays

Is it an antibiotic resistant TB? **MDR/XDR detection**

- **Molecular tests:**
  - GeneXpert® MTB/RIF, Line probe assays, target genes sequencing
- **Phenotypic drug susceptibility testing**
Phenotypic susceptibility testing

First, second and third line antibiotics

• Classic proportions method LJ medium
• Faster liquid medium MGIT
Liquid Medium Phenotypic Susceptibility Testing

BD Bactec™ MGIT™ 960

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Low concentration (mg/L)</th>
<th>High concentration (mg/L)</th>
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</thead>
<tbody>
<tr>
<td>STR</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>INH</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>RIF</td>
<td>1.0</td>
<td>-</td>
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<tr>
<td>EMB</td>
<td>5.0</td>
<td>7.5</td>
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<tr>
<td>PZA</td>
<td>100</td>
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<tr>
<td>FQ, AMK</td>
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</tbody>
</table>
Liquid Medium
Phenotypic Susceptibility Testing

BD Bactec™ MGIT™ 960
Phenotypic Susceptibility Testing: Reference Proportions Method

- 1961 by Canetti, Rist and Grosset
- Numeration of the surviving colonies by comparison to the tube without antibiotics
- Sensitive strain: <1% survivor
- Reading of results starting at 21 days
- Second reading 15 days later
Solid Medium Phenotypic Susceptibility Testing

Proportions method

Löwenstein-Jensen medium filled with antibiotics in various concentrations:

- Isoniazid (0.1, 0.2, 1 and 10 mg/L)
- Rifamycine (40 mg/L)
- Ethambutol (2 mg/L)
- Streptomycine (4 mg/L)
- Fluoroquinolones, amikacine, …
Phenotypic Susceptibility Testing
Proportions Method

<table>
<thead>
<tr>
<th></th>
<th>Control without ATB</th>
<th>Control without ATB</th>
<th>INH 0.1µg/ml</th>
<th>INH 0.2µg/ml</th>
<th>INH 1µg/ml</th>
<th>RIF 40µg/ml</th>
<th>EMB 2µg/ml</th>
<th>STR 4µg/ml</th>
</tr>
</thead>
</table>

Sensitive strain
MDR (rpoB S531L, INH$_R^R$ katG S315T) EMB$_R^R$

<table>
<thead>
<tr>
<th></th>
<th>INH 0.1 mg/L</th>
<th>INH 0.2 mg/L</th>
<th>INH 1 mg/L</th>
<th>INH 10 mg/L</th>
<th>RIF 40 mg/L</th>
<th>EMB 2 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Image of test tubes with various antibiotic concentrations](image-url)
Timelines for diagnostic testing

- **Week 0**: Microscopy - AFB
- **Week 1**: Molecular assays - RIF<sup>R</sup> (= 95% MDR)
- **Week 2**: Liquid Culture
- **Week 3**: Phenotypic DST
- **Week 4**: NGS
- **Week 5** to **Week 12**: MDR/XDR
谢谢
Further slides are for potential questions
In Rapid implementation of the Xpert MTB/RIF diagnostic test, OMS 2011
Xpert MTB/RIF

RIF-R prevalence

PPV
NPV

In Rapid implementation of the Xpert MTB/RIF diagnostic test, OMS 2011
TB LAMP test

Loop-mediated isothermal amplification

Prepare lysate
Dried lamp reagents
Add 30 µL mix
40 min / 67°C

Fluorescent signal detection

< 1 hour to detect MCTB (urines in HIV patients)
No sophisticated instrument
Training, electricity, temperature < 30°C