Bacteriological and Molecular Diagnosis of Childhood TB in Low / Intermediate Burden Settings

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Andreas Roth
Anne Detjen

Berlin, Germany
Microbiological Diagnostic Tools

Microscopy

Culture

PCR

Array technique (chip)
Microbiological Diagnostics
Ziehl Neelsen Staining/Fluorescence microscopy

Detection limit: $10^4$ bacteria/ml
Turn-around time: 20 min
Test sensitivity: ~ 20% (~30%*)

*fluorescence microscopy

Lighter J, Rigaud M
Diagnosing childhood tuberculosis: traditional and innovative modalities.
Microbiological Diagnostics - Culture

Gold standard (LJ) – high specificity

Detection limit 10-100 bacteria/ml
Turn-around time: liquid medium (~14 days) solid medium (~ 4 weeks)
Sensitivity: liquid ~ 50% solid ~ 30-40%

MGIT 960

Lighter J, Rigaud M
Diagnosing childhood tuberculosis: traditional and innovative modalities
Microbiological Diagnostics

**PCR (NAAT*)**

Detection limit: ~ 10 bacilli
Turn-around time: ~ 24-48 h
Sensitivity:
- 40-60% in smear negative but culture positive
- 90-100% in smear positive and culture positive

*NAAT = Nucleic Acid Amplification Techniques

Gomez-Pastrana D
*Tuberculosis in children—is PCR the diagnostic solution?*
*Clin Microbiol Infect* 2002 Sep 8:541-544
Clinical Specimen

N-Acetyl-L-Cystein-NaOH (NALC) decontamination
DIN 58943-3

Methods for direct detection

Culture
solid medium

Culture
broth

Smear

Microscopy

PCR

COBAS®
AMPLICOR® MTB

In case of growth

culture confirmation and molecular biological identification/typing

Microscopy

Subculture

Hybridisation probe
(Gen Probe®)

RFLP
(Fingerprint)

PCR (ID)
16S rDNA

 Susceptibility

Biochemical typing

PCR Susceptibility

Bacteriological Workflow
Clinical Specimen

N-Acetyl-L-Cystein-NaOH (NALC) decontamination
DIN 58943-3

1,5 ml

Methods for direct detection

Culture
solid medium

Culture broth

Smear

10 µl

Microscopy

100 µl

PCR

COBAS® AMPLICOR® MTB

pos

In case of growth

culture confirmation and molecular biological identification/typing

Microscopy

PCR (ID)
16S rDNA

RFLP
(Fingerprint)

Hybridisation probe
(Gen Probe®)

Subculture

Biochemical typing

Susceptibility

PCR Susceptibility
**PCR: Molecular susceptibility testing**

**Lineprobe Assay**

- **Hain Genotype MTBDR**
  - robust
  - reliable
  - low price
  - labour intensive „washing“
  - max. 18 samples

**INNO-LiPA Rif.TB assay**

- RMP resistance
- INH resistance
Array technique for identification and susceptibility testing (chip technology)
Chip (technology): low cost / low density array
Suceptibility testing: Chip *Myco-Res*

**Turn-around time:** ~ 4 h

<table>
<thead>
<tr>
<th></th>
<th>rpoB</th>
<th>katG</th>
</tr>
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<tr>
<td>RMP susceptibility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH susceptibility</td>
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**Chip Details:**

1. WT-AA-504-509
2. WT-AA-509-515
3. WT-AA-514-519
4. WT-AA-520-524
5. WT-AA-525-530
6. WT-AA-530-534
7. Mut-511-Pro
8. Mut-512-Thr
9. Mut-516-Tyr
10. Mut-516-Val
11. Mut-526-Asn
12. Mut-526-Leu
13. Mut-526-Asp
14. Mut-526-Tyr
15. Mut-526-Arg
16. Mut-531-Leu
17. Mut-531-Trp
18. Mut-533-Pro
19. WT-315-Ser (katG)
20. Mut-315-Thr (katG)
21. Mut-315-Asn (katG)
Suceptibility testing: Chip Myco-Res

Sputum (AFB +)

**rpoB**: WT  
**katG**: WT

**rpoB**: 516-Tyr  
**katG**: WT

**rpoB**: 526-Asp  
**katG**: WT

**rpoB**: 531-Leu  
**katG**: 315-Thr

Capture probe for mutant 516-Tyr positive global: 3.9 %

Capture probe for mutant 531-Leu positive global: 43.2 %
### Identification: Chip *Myco-Direct 1.7*

#### Turn-around time:
- ~ max. 4 h

#### e.g. sputum (AFB +)

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<thead>
<tr>
<th>Nr.</th>
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<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Mycobacteria Group II</td>
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<td>3</td>
<td>Mycobacteria Group III</td>
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<td>4</td>
<td>M. tub complex 01</td>
<td>A</td>
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<tr>
<td>5</td>
<td>M. tub complex 02</td>
<td>B</td>
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<td>6</td>
<td>M. avium complex 01</td>
<td>A</td>
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<td>7</td>
<td>M. avium complex 02</td>
<td>A</td>
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<tr>
<td>8</td>
<td>M. kansasii 01</td>
<td>A</td>
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<td>9</td>
<td>M. kansasii 02</td>
<td>A</td>
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<tr>
<td>10</td>
<td>M. xenopi</td>
<td>A</td>
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<td>11</td>
<td>M. abscessus</td>
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<td>M. gordonae</td>
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<td>15</td>
<td>M. haemophilum</td>
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<td>16</td>
<td>M. marinum/ulcerans</td>
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<td>M. simiae</td>
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<td>18</td>
<td>M. smegmatis</td>
<td>A</td>
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<tr>
<td>19</td>
<td>Internal Standard</td>
<td>optional</td>
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<tr>
<td>C</td>
<td>Hyb-Control</td>
<td>-</td>
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Clinical basic application

- Up to date recommendation -
CDC Updates Guidelines for Nucleic Acid Amplification Techniques to Diagnose Tuberculosis

Laurie Barclay

*Morb Mortal Wkly Rep* 2009 58:7–10

- NAAT results should be interpreted in conjunction with the AFB smear results.

- NAAT and smear positive: start Rx despite pending culture results. PPV 95%

- Smear negative, NAAT positive: use clinical judgment to either treat or await culture
Selection from automated systems for molecular and bacteriological rapid diagnostics

**PCR:**

Roche/COBAS®: Amplicor® amplification kits

Roche/COBAS®: LightCycler® (real-time-PCR)

Roche/COBAS®: TaqMan 48®

(increases the specificity of real-time-PCR)

**Culture:**

BD Bactec™ MGIT* 960:

bacteriological broth diagnostics

* Mycobacteria Growth Inhibitor Tube
Workflow: Diagnosis of Tb using microscopy, culture, and NAAT

- **Acute disease or high suspicion**
  - TST, IGRA
    - **All positive**
      - Clinical signs & symptoms, risk
      - **Microscopy and culture and NAAT**
        - Microscopy positive or negative culture and/or NAAT positive
          - Active disease
Workflow: Diagnosis of Tb using microscopy, culture, and NAAT

Why still microscopy?
- cheap
- easy
- fast
- semiquantitative
- specificity?
- in combination with PCR 100% specificity

1. acute disease or high suspicion
2. TST, IGRA
3. all positive
   + clinical signs & symptoms, risk
   ⇒ microscopy and culture and NAAT
4. microscopy positive or negative culture and/or NAAT positive
   ⇒ active disease
Summary

1. Principle methods for TB diagnostics are: microscopy, culture, and PCR

2. PCR can't yet replace neither microscopy nor culture but it compliments both methods

3. No testing method replaces clinical assessment
Traditional and Modern TB-Diagnostics

Together they are strong
Workflow: Diagnosis of Tb using microscopy, culture, and NAAT

- **Suspicion**
  - No clinical signs & symptoms
    - All negative: Healthy
    - All positive: LTBI
  - TST, IGRA
    - TST positive, IGRA negative
      - All positive: NTM?
      - All negative: Active disease
    - All positive: Active disease
  - Clinical signs & symptoms, risk
Thank you for your attention!
Rapid diagnostics? …

... save time and money
(i.e. MDR direct detection)

... still no readiness to use
so-called “expensive” PCR-tests
Workflow: Diagnosis of Tb using microscopy, culture, and NAA

- **Acute disease**
- **High suspicion**
- **“Exclusion”**

Microscopy and culture and NAA

Microscopy positive, culture and/or NAA negative

- **No clinical signs & symptoms, no risk**
- **Clinical signs & symptoms, risk**

- **Healthy**
- **LTBI**
- **NTM?**
- **Active disease**
Workflow: Diagnosis of Tb using microscopy, culture, and NAA

- **Acute disease or high suspicion**
  - **Microscopy and culture and NAA**
  - **Microscopy negative, culture and/or NAA positive**
    - **No clinical signs & symptoms, no risk**
      - Healthy
    - **Clinical signs & symptoms, risk**
      - LTBI
      - **NTM?**
      - Active disease
Capture probe
WT amino acids 514-519

Capture probe for
mutant 516-Tyr positive
global: 3.9 %

Capture probe
WT amino acids 530-534

Capture probe for
mutant 531-Leu positive
global: 43.2 %
1990 Eisenach: PCR IS6110
1990 Böttger: 16S RNA Gene
1993 Telenti: RFLP hsp Gene
1993 Telenti: rpoB Gene
1995 Amplicor/ MTD
1999/2001 MIQ NAA
1990 Böttger: 16S RNA Gene
1990 Eisenach: PCR IS6110
1993 Telenti: RFLP hsp Gene
1995 Amplicor/ MTD
1999/2001 MIQ NAA
1990 Böttger: 16S RNA Gene
1990 Eisenach: PCR IS6110
1993 Telenti: RFLP hsp Gene
1995 Amplicor/ MTD
1999/2001 MIQ NAA
Capture PCR
A PCR strategy in which linkers added to the ends of linear DNA molecules are used as primer binding sites and intramolecular stem-loop structures are exploited for strand specific priming. The products generated by amplification of the sequence between the two primers correspond can be captured by streptavidin.

Capture probe
A phage or antibody probe that binds proteins in a sample enabling relative expression levels to be detected.
infiltration/cavern
abscess
MDR-risk
immunosuppression

probe

SFS
GPS
mycel

culture sensitivity
(phenotype)

PCR

MTB
MOTT
Noc
RIF 90%
INH 70%
<table>
<thead>
<tr>
<th>Method</th>
<th>n</th>
<th>(%)</th>
<th>„simple“</th>
<th>vs all samples</th>
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<td>Amplicor</td>
<td>54</td>
<td>46.6%</td>
<td>99 %</td>
<td>89 % (-10)</td>
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<td>57</td>
<td>58.8%</td>
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<td>91 % (-5)</td>
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<td>GenProbe</td>
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<td>8.6%</td>
<td>100 %</td>
<td>94 % (-6)</td>
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<td>8.5%</td>
<td>98 %</td>
<td>94 % (-4)</td>
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<td>In-house</td>
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<td>24.1%</td>
<td>96 %</td>
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<td>19.8%</td>
<td>96 %</td>
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<td>ProbeTec</td>
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<td>17.2%</td>
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<td>97 % (-0)</td>
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<td></td>
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<td>13.2%</td>
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<td>97 % (-2)</td>
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<td>3.4%</td>
<td>100 %</td>
<td>82 % (-18)</td>
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<td></td>
<td>5</td>
<td>4.7%</td>
<td>88 %</td>
<td>83 % (-5)</td>
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TB PCR
Genus PCR
in house
Amplicor
LightCycler - Arrays
Genus PCR
TB PCR
LightCycler - Arrays
sensitivity
culture
chip technology
microscopy
differentiation
identification
resistance
Multigenotypic post-PCR analysis of multiple specificities

Microarrays
Chiptechnology with fluorescence not routinely used for a clinical settings yet.
Differentiation of Mycobacteria

- conventional (growth, colour, form, biochemistry)  
  2 - 4 weeks or longer

- molecular (gene probes, PCR, sequencing 16S rRNA)  
  2 - 7 days

<table>
<thead>
<tr>
<th>Mycobacterium</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Sequence 3</th>
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<tr>
<td><em>M. tuberculosis</em></td>
<td>TAAAGC</td>
<td>GCCTTCACCA</td>
<td>CATGCA</td>
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<td><em>M. Intracellular</em></td>
<td>AAAAA--</td>
<td>GCCTTCACCA</td>
<td>CATGCA</td>
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<td><em>M. chelonae</em></td>
<td>AAA---</td>
<td>GCCTTCACCA</td>
<td>CATGCA</td>
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<td><em>M. smegmatis</em></td>
<td>AAA---</td>
<td>GCCTTCACC</td>
<td>CATGCA</td>
</tr>
<tr>
<td><em>M. xenopi</em></td>
<td>TACCAAC</td>
<td>GCCTTCACCA</td>
<td>CATGCA</td>
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</tbody>
</table>
Hand in Hand

Tradition and Hightech in TB Diagnostics
Bacteriological Tuberculosis-Susceptibility Testing

- solid medium
  *pure culture + 4 weeks*

- broth
  *pure culture + 1 week*

- molecular (PCR)
  *2 - 3 days*

**ATTENTION:**
Detection rate of resistant strains:
rpoB 98%, katG 70%
Diagnostic Tools: Assessment and Recommendations

Culture

- „Gold standard“
  most sensitive and specific

- Solid medium: limit of detection 100 bacteria, growth of TB after 4 weeks

- Broth: limit of detection 10 bacteria, growth of TB after ~2 weeks (e.g. MGIT®-System: Mycobacteria Growth Inhibitor Tube)
For example:
Rapid diagnosis of tuberculous meningitis: a comparative evaluation of in-house PCR assays involving three mycobacterial DNA sequences, IS6110, MPB-64 and 65 kDa antigen
Rafi W et al.
J Neurol Sci  2007

Against a gold standard of culture, a sensitivity of 98% (NPV=99%) and a specificity of 100% (PPV=100%) was observed with the IS6110 PCR. Among the nested PCRs, a sensitivity of 91% (NPV=94%) and a specificity of 91% (PPV=85%) was observed with the MPB-64 assay.
Real-Time-Sensitivity?

Comparison of real-time polymerase chain reaction using the Smart Cycler and the Gen-Probe amplified Mycobacterium tuberculosis direct test (MTD) for detection of M. tuberculosis complex in clinical specimens

Pounder JI, Aldous WK, Woods GL
Diagn Microbiol Infect Dis 2006

Real-Time: Sen 86.3% Spez 100% PPV 100% NPV 94.5%
MTD: Sen 98.0% Spez 99.2% PPV 98.0% NPV 99.2%

Comparison of an internally controlled, large-volume LightCycler assay for detection of Mycobacterium tuberculosis in clinical samples with the COBAS AMPLICOR assay

J Clin Microbiol. 2005

Sensitivity COBAS Amplicor = Real-Time

TaqMan Roche?
Workflow: Diagnosis of Tuberculosis using PCR

- **acute disease**
- **high suspicion**
- **"exclusion"**

**Microscopy and culture and NAA**

- **AFB positive**
- **negative**

**TB-NAA**

- **TB-Nucleic acid amplification (NAA)**
  - negative
  - not reproduc
c

**clinical decision → await culture**

**Diagnosis**

- Tuberculosis
- await culture
- no NAA

**NTM ?**

- **TB positive**
Indication for PCR/ NAA Mycobacteriology

Sensitivity: culture (100%) ≥ PCR (~90%) >> smear (~50%)

Approved indication (routine laboratory)

• Respiratory tract secretions (tuberculosis)
  1. Microscopy positive (and AIDS)
  2. Microscopy negative and clinical suspicion of tuberculosis

• CSF
  3. Meningitis tuberculosa

Special indications (reference laboratory)

• Extrapulmonary tuberculosis
• NTM