LAB METHODS: TUBERCULOSIS DIAGNOSIS

TB CASE MANAGEMENT AND CONTACT INVESTIGATION INTENSIVE
OCTOBER 8-11, 2019

LEARNING OBJECTIVE

Upon completion of this session, participants will be able to:
1. Utilize rapid identification methods for drug susceptibility for tuberculosis to improve patient outcomes

INDEX OF MATERIALS

- Lab methods: tuberculosis diagnosis slide outline
  
  Presented by: Grace Lin, MS

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ADDITIONAL REFERENCES

• Barnes, P., Cave, D. Molecular epidemiology of tuberculosis. NEJM. 2003;349(12): 1149-1155. Review article.
Laboratory Methods:
Tuberculosis Diagnosis

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10/8/19

Mycobacterium tuberculosis Complex (MTBC)

- M. tuberculosis
- M. bovis
  - M. africanum
  - M. caprae
  - M. microti
  - M. pinnipedii
  - M. mungi, M. orygis, M. canettii, M. suricattae (proposed)

All cause TB
MTBC

- Acid-fast bacilli (AFB)
  - NTM (non-TB-mycobacteria) are also AFB
  - When AFB smear is positive, it can be MTBC or NTM
- Slow growing
  - Easily be overgrown by other bacteria
  - Require special procedures to process specimen
    - Decontamination and concentration → sediment
  - Delayed turnaround time (TAT)
    - Molecular testing significantly shortens TAT

Specimen

Sputum: the most common specimen type

- Non-sterile source
  - Rinse month with water before collecting
  - First morning, deep cough sample—best yield
  - 3-5 ml, collect in sterile, leak-proof containers
  - Refrigerate if cannot send to lab immediately
- For diagnosis: 3 samples (CDC), 2 (WHO)
  - Collect on different days or at least 8 hr apart.

Other specimen types:
  BAL, pleural fl, lymph node, other tissues, CSF, etc
Case 1

- 60 yr Chinese male came to U.S. to attend his son’s PhD graduation
- Cough x 3+ weeks
- Lose weight? (pt said, no, no...)
- A sputum sample was collected for AFB smear & culture at Alameda PH lab
Specimen Processing

- Liquification by NALC
- Decontamination by NaOH
- Concentration by centrifugation

AFB Smear

- Fluorochrome stains
  - AR (Auramine-rhodamine)
    - Fluorescent microscope.
    - Golden orange-yellow rods
    - More sensitive than ZN or Kinyoun.

- Carbo fuchsin-based stains
  - ZN (Ziehl-Neelsen) or Kinyoun stain
    - light microscope.
    - AFB--red. Non-AFB--blue
Case 1

• Smear: AFB negative
• Let’s ask Dr. Chen if a molecular test should be ordered?
  • Yes! Please run Xpert.
    • FDA approved for testing sputa, smear-pos or neg
    • But, test performance for smear-neg samples is not as good as for smear-positive samples.

Xpert MTB/RIF Assay
Xpert MTB/RIF assay

- A great molecular test for:
  - MTBC detection and RIF-R detection
  - Cannot differentiate M. tb from M. bovis

- Realtime PCR, 5 molecular beacon probes, A to E
  - rpoB gene—81 bp core region
    - >95% of RIF-resistant strains have mutations in rpoB
    - Assuming if mutations detected → RIF-R
      - Yes, most times, but not always.

Molecular Beacon probe
(Hairpin structure—head & two arms)

- Head (MTBC sequence)
- Two arms (5-7 nt)

At “rest” stage, two arms bind together forming a stem. Fluorophore is quenched. No signals are produced.
Mutation Detection with Molecular Beacons
(Head containing wildtype SQ)

Mutant Sequence

Wildtype Sequence

MB at rest

MB in action

MB’s head does not bind to mutant SQ.
Arms remain closed.
No signals produced.

MB’s head binds to wildtype SQ.
Arms open. Fluorophore is away
from quencher. Signals produced.

Critical Rules for Interpretation
(Set by the Xpert software)

• MTBC detected
  – If 2 or more probes have signals = MTBC

• RIF-R detected
  – If a probe does not generate signals (Ct = 0), or
  – If the highest and lowest Ct differs by more than 4 (Δ Ct max > 4)

• See more rules in package insert
**Xpert Clinical Report**

Procedure: MTB complex Nucleic Acid Amplification Test  
Source: sputum  
Final report (4 possibilities):  
1. MTB complex not detected by PCR  
2. MTB complex detected by PCR. RIF resistance not detected  
3. MTB complex detected by PCR. RIF resistance detected  
4. Detection of MTB complex was indeterminate

**Case 1**

**Xpert Clinical Report**

Procedure: MTB complex Nucleic Acid Amplification Test  
Source: sputum  
Final report: MTB complex not detected by PCR
Xpert Raw Report

- Hidden information in raw reports
  - Does SPC (specimen control) have signals?
  - How many probes are up (having signals)?
  - MTBC detected because how many probes are up?
  - RIF-R detected by which probe(s)?
  - Ct values?

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<table>
<thead>
<tr>
<th>Ct</th>
<th>End-pt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe D</td>
<td>0</td>
</tr>
<tr>
<td>Probe C</td>
<td>0</td>
</tr>
<tr>
<td>Probe E</td>
<td>0</td>
</tr>
<tr>
<td>Probe B</td>
<td>0</td>
</tr>
<tr>
<td>SPC</td>
<td>24.9</td>
</tr>
<tr>
<td>Probe A</td>
<td>0</td>
</tr>
</tbody>
</table>

SPC has signals:
- No inhibitory substances.
- No probe has signals
  - MTBC: Not detected
- Does NOT rule out MTBC
  - MTBC DNA too low
  - No MTBC DNA (NTM?)
- Wait for culture to grow or test another specimen
Ask Dr. Chen for advice

• Test another sputum.

• MMWR 2/27/15
  • For smear-neg specimens, if test 2 sputa, sensitivity increases from 55% to 69%.

Collect another sputum

• Jonny sets off to collect another sputum
  • He thinks how to help lab to produce better results....
  • A better specimen would help!!

  • Quality
    • First morning, deep deep cough
  • Quantity
    • 5 ml
  • Send to lab ASAP or refrigerate. (Why?)
Ct values are high
- DNA: low
- Smear: negative
5 probes up
- MTBC detected
- No mutation
- Δ Ct max < 4
  \((33.9 - 31.8 = 2.1)\)
- RIF-5

<table>
<thead>
<tr>
<th>Probe</th>
<th>Ct</th>
<th>End-pt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe D</td>
<td>33.9</td>
<td>88.0</td>
</tr>
<tr>
<td>Probe C</td>
<td>32.6</td>
<td>115.0</td>
</tr>
<tr>
<td>Probe E</td>
<td>32.9</td>
<td>113.0</td>
</tr>
<tr>
<td>Probe B</td>
<td>33.6</td>
<td>73.0</td>
</tr>
<tr>
<td>SPC</td>
<td>29.7</td>
<td>232.0</td>
</tr>
<tr>
<td>Probe A</td>
<td>31.8</td>
<td>89.0</td>
</tr>
</tbody>
</table>

No RIF resistance detected. Great!

The 2\(^{nd}\) sample worked!
It was a better specimen.

What if the 2\(^{nd}\) sample did not work?
* MTBC may be present but too few cells
* Detection limit ~ 100 colonies/mL
* Could be NTM/other microbes.....
Worst false results by Xpert (when testing smear-negatives)

- Two probes positive = MTBC – a problematic rule
- NTM may be mis-identified as MTBC with RIF-R
  - 2 probes have low signals barely cross the threshold
  - Means 3 probes are negative (no signals) = at least 3 mutations
  - RARE to have 3 mutations within 81 bp of rpoB core
  - Of 3754 tested, 2 samples had 3 mutations (CA data)
    - None detected by 3 probes
    - One detected by 2 probes (A & B)
    - One detected by 1 probe (D)

Next day, another TB suspect walks in…….
Case 2

• 82 yr male, US-born, no travel hx
• Cough x 4 weeks, weight loss
• No prior TB treatment
• Smear negative, culture pending
• Shall Xpert be ordered?
  – Yes
  – RIF-R suspected? No…..

Xpert Clinical Report

Procedure: MTBC nucleic acid amplification test
Source: sputum
Final report:
  MTB complex detected by PCR
  Rifampin resistance detected

What?!
MUST Ask lab for Xpert raw report.
When Xpert detects RIF resistance

- Submit for sequencing
  - CA PSQ or CDC MDDR services
  - Verify if rpoB mutations are present
  - Identify the Mutation SQ if present
    - Silent or missence?
    - Disputed mutation? (Mutations conferring phenotypic R)
    - Predict levels of resistance to RIF & RFB*
- Additional molecular results
  - INH and 2nd-line drugs
  - Helpful for formulating treatment regimens

PSQ Detected a Silent Mutation

• 514TTT (433TTT using MTB codon)
  – Great! It does not confer RIF-R.
  – The most common silent mutation
    • Found in ~70% of the mutations detectable by probe B
  – It was interpreted as RIF-R by Xpert
    • Xpert detects presence/absence of mutations, does not know if it is silent
  – Other mutations detected by Probe B may confer RIF-R
    • Before SQ results are available, evaluate RIF-R risk factors

Another TB suspect......
Case 3

• 40 yr female from Philippines
• A nurse assistant works at SFGH
• Cough x 4 weeks, weight loss
• 10 yr ago treated for TB
• A sputum sample was collected
  • Smear 1+, culture pending
• Xpert test was ordered

Xpert Clinical Report

Procedure: MTBC nucleic acid amplification test
Source: sputum
Final report:
  MTB complex detected by PCR
  Rifampin resistance detected

Not Surprised, unfortunately!
Ask lab for Xpert raw report.
4 probes up
- MTBC detected
- Probe E: no signal
- Mutation detected
- RIF-R detected
- Probe E detects most common mutation in MDR

Actions
- Send for sequencing

Pyrosequencing (PSQ)
- Realtime sequencing
- TAT: 1 day
- Can detect
  - MDR:
    - R to INH & RIF
  - XDR:
    - R to INH, RIF, fQs, injectable drugs (AK/CM/KM)
Criteria for Requesting PSQ

- Confirmation of RIF-resistance detected by Xpert.
- Drug-resistant TB is suspected
  - Immigrants from countries of high DR prevalence
  - Contact of DR patients
  - Previously treated cases / Not responding to treatment
- Patients have wide exposure or to vulnerable population
- Patients with vulnerable conditions (HIV+, transplant, etc.)
- Patients have adverse reactions to INH or RIF
  - Need to treat with 2\textsuperscript{nd}-line drugs
- Laboratory issues
  - Smear-pos but culture-neg for undiagnosed TB
  - Mixed cultures, cDST cannot be performed
  - Confirmation of DR from culture-based DST
PSQ of *pncA* for Identification of M. bovis

- Differential identification of:
  - M. bovis
  - MTBC-not-M. bovis
- M. bovis is PZA-R
- ID of M. bovis before culture grows
  - Avoid PZA treatment
MDDR at CDC

- Require pre-approval
- Able to detect XDR. Molecular targets are:
  - INH: *katG* & *inhA*.
  - RIF: *rpoB*
  - EMB: *embB*
  - PZA: *pncA*
  - fQ: *gyrA*
  - Injectable drugs: *rrs, eis, tlyA*

mDST

<table>
<thead>
<tr>
<th>Specimen</th>
<th>GeneXpert</th>
<th>PSQ (MDL)</th>
<th>Sanger (CDC)</th>
<th>Targeted NGS (tNGS)</th>
<th>WGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum, Raw sample or sediments</td>
<td>All sources sediments or cultures</td>
<td>All sources sediments or cultures</td>
<td>All sources sediments or cultures</td>
<td>All sources sediments or cultures</td>
<td>Pure cultures</td>
</tr>
<tr>
<td>TAT</td>
<td>2-3 hours</td>
<td>1-2 days</td>
<td>1-2 days</td>
<td>4-5 days (~1 week)</td>
<td>4-5 days (~1 week)</td>
</tr>
<tr>
<td>Drug</td>
<td>RIF only</td>
<td>INH, RIF, fQs, AMK, CAP</td>
<td>INH, RIF, EMB, PZA fQs, AMK, CAP, KAN</td>
<td>Any drug if genetic targets are known</td>
<td>Any drug if genetic targets are known</td>
</tr>
</tbody>
</table>
| Results         | Mutations present or not. (Mutation identity not provided) | Wildtype or mutant sequences provided (Hetero-R can be detected but not as sensitive as tNGS) | Wildtype or mutant sequences provided (Hetero-R can be detected but not as sensitive as tNGS) | Wildtype or mutant sequences provided | Wildtype or mutant sequences provided (Hetero-R (Not as reliable sensitive as tNGS) 3

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Workflow

Specimen

Sediment

Processing

Smear

Molecular Testing
- Xpert
- PSQ, MDDR

Culture

MTBC

NTM

cDST

Genotyping

Specimen processing

1 day

6 weeks

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Culture

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TB Case Management and Contact Investigation Intensive
Oakland, CA
October 8-11, 2019
To assure better recovery, liquid & solid media should be inoculated for primary isolation.
- Different media provide different nutrients

**Liquid media**
- MGIT (BD)
- BacT/Alert (bioMerieux)
- Myco (VersaTrek)

**Solid media**
- Egg-based: LJ
- Agar-based: Middlebrook 7H10, 7H11

- Plates: allow to view colonial morphologies to discern MTBC, NTM or non-ABF, also possible to pick colonies if mixed

Liquid medium instruments Continuously monitor growth.
Detect growth faster than solid media

MGIT 960

MGIT tubes fluoresce when O\(_2\) is reduced/depleted.
MTBC

Rough colonies on 7H10

Cellular Morphology
(seen on smears made from positive cultures)
MTBC Cording Clumps
(seen on smears made from positive cultures)

Early cording clumps
Older cording clumps

Colonial Morphology
(Microscopic)

MAC
MTBC
Culture Identification

- Cultures take 2-3 weeks to grow (smear 4+, may grow in 1 wk)
- DNA probes (AccuProbe)
  - MTBC
  - M. kansasii
  - MAC
  - M. gordonae
- MALDI-TOF (mass spectrometry)
  - Matrix assisted laser desorption ionization-time of flight
  - Protein profiles
- Conventional methods
  - pigmentation, biochemical tests, growth rate, etc.
- DNA sequencing

These 4 probes identify ~90% of AFB isolated in clinical labs.

CDST

Must set up from pure cultures

- Agar proportion (“conventional”)—21 days
- Modified proportion methods using liquid media
  - MGIT (4-14 days, average 7-8 days)
  - VersaTrek (4-13 days)
- Sensititre microdilution MIC method, (7 to 21 days)
  - Not FDA approved yet
  - Use growth from solid media to test, delayed TAT.
  - Not available for testing PZA
  - Drug panels can be customized for testing BDQ, LZ CF, etc.
Interpretation for Resistance Agar Proportion method (used at CDC)

• Resistant:
  – growth in drug quadrant ≥ 1 % of growth in the control (with no drug).
• SIRE, ETA, KAN, OFX, RFB, PAS. (PZA by MGIT).

MGIT DST at MDL

• 1<sup>st</sup>-line: RIEP
• 2<sup>nd</sup>-line: MACE (MFX, AMK, CAP, ETA)
• Plan to validate for testing LZN, BDQ
**Verification of Resistance detected by MGIT**

- Visual check MGIT tubes
  - MGIT may appear turbid due to contamination.
- Quick confirmation of R by sequencing
  - Mutations detected \( \rightarrow \) R results are confirmed
  - Mutations not detected, retest from a pure culture.
- Review purity plate
  - Even if culture is pure, contamination may be introduced during DST set-up. Need to check smear morphology on MGIT/VT showing R.
- Check smear morphology
  - To differentiate MTBC vs NTM
  - If smear morphology is not clear-cut, make sub onto 7H10 or 7H11 plates. Check colonial morph in a few days.

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**When you have doubts in DR results**

- It is critical to communicate with lab
- Ask lab how they verify the DR results
  - DST must be set up from pure culture
  - Insidious presence of NTM may not be detected initially
  - Contamination may be introduced during set-up
When lab testing is not successful

• When need to retest, find out why, get a better sample
• No sequences yielded / MTBC not detected by Xpert
  – MTBC DNA too low or not present
  – If MTBC is strongly suspected, get a better specimen: deep cough sputum, higher volume.
  – If MTBC is not strongly suspected, wait for culture to grow. pt may be infected with NTM/other microbes.
• If culture is contaminated
  – When collecting another sputum, make sure pt rinses mouth thoroughly with clean water (bottled water)
  – Deliver sample to lab ASAP or refrigerate.

Time Frame for Results

<table>
<thead>
<tr>
<th>Test</th>
<th>Testing Time</th>
<th>Turnaround Time (from date rec'd at lab performing tests)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB Smear</td>
<td>1 hr</td>
<td>1 day</td>
</tr>
<tr>
<td>Xpert</td>
<td>3 hr</td>
<td>1-2 days</td>
</tr>
<tr>
<td>PSQ</td>
<td>6 hr</td>
<td>1-2 days</td>
</tr>
<tr>
<td>MDDR (CDC)</td>
<td>1 day</td>
<td>1-2 days</td>
</tr>
<tr>
<td>Culture Positive</td>
<td>6 wk incubation</td>
<td>Avg. 2-3 wk for positives 6 wk for negatives</td>
</tr>
<tr>
<td>ID by Accuprobe</td>
<td>1.5 hr</td>
<td>3-4 wk (tested once/wk)</td>
</tr>
<tr>
<td>Susceptibility</td>
<td>1-3 wk</td>
<td>4-8 wk (tested once/wk)</td>
</tr>
</tbody>
</table>
Genotyping

- Epidemiology surveillance
- Investigation of outbreaks
- Investigation of cross-contamination
- Differential diagnosis of relapse, reinfection, specimen collection error, or lab errors

Case 4

* 5 years ago, a 45 yr old male from Mexico was treated for pan-S TB. At 6 wks into treatment, culture was converted and remained negative at the completion of treatment.

* Now, MTBC was isolated and tested R to RIPE by MGIT.

** Quite unusual, what happened?

- False DR? (mixed or contaminated?)
  - No, culture was pure
- Wrong isolate? No, another current isolate yielded same results
- Acquired resistance or infected with a different strain?
- Let’s see Genotyping results.........[to be continued....]
Genotyping results (cont.)

- If the current and old isolates have **different** genotypes
  - Reinfected with a different strain
- If the current and old isolates have the **same** genotype
  - If the genotype is rare, likely it is acquired-R
    - A possibility is that the uncle acquired-R and he reinfected with the uncle’s
  - If the genotype is very common,
    - Possibly reinfected with a different strain which has the same genotype. [WGS may have strong power to sort this out.]

Case 5

- A 64 yr old female came from Taiwan 40 years ago to attend a graduate school.
- She was seen by a family doctor at a private hospital because of coughing for more than 4 weeks.
- 3 sputa were collected and sent to a commercial lab for AFB & culture work-up. All 3 smear were negative.
- 6 weeks later the family doctor received a lab report stating M. tuberculosis complex isolated, Drug susceptibility testing pending. Other 2 sputa yielded no growth.
- Title 17 requires reporting of TB. The case was forwarded to a county TB control program.
  - **Something wrong?**
Thank you!