Harmonizing the Use of Molecular & Culture-based DST of *Mycobacterium tuberculosis*

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Harmonizing?

• There must be some conflicts?
• Yes!
• Nothing is perfect, but each has its strength!
• How to make best of both methods to achieve the goal of providing timely, accurate DST results to clinicians—that is the work of harmonizing!
Disclaimer

- No financial affiliation with the companies whose products will be discussed in this presentation.
Acronyms

• **DST**: Drug susceptibility testing
• **MDST**: Molecular DST
• **CDST**: Culture-based DST
• **AP**: agar proportion method
• **DR/DS**: drug resistance/susceptibility
• **SQ**: sequencing or sequence
• **TAT**: turnaround time
What are not in “harmony”?

- MDST detected no mutations; CDST tested R.
- MDST detected a mutation; CDST tested S.
- Discrepancies occur:
  - between CDST methods: AP & MGIT disagree
  - between labs or within lab, by different methods or even the same method.
- Discrepancies between MDST methods.
- Which result to trust? Headaches for clinicians!
## Comparison

<table>
<thead>
<tr>
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<th>MDST</th>
<th>CDST</th>
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<tr>
<td><strong>TAT</strong></td>
<td>Fast (1-3 days)</td>
<td>Slow (weeks)</td>
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<tr>
<td><strong>Availability</strong></td>
<td>Limited drugs</td>
<td>Most drugs</td>
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<tr>
<td><strong>Requires pure and viable cultures</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Sensitivity &amp; Specificity</strong></td>
<td>Not 100%</td>
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MDST--benefits

• Quick screening for DR
  – No mutations detected, it provides good prediction for DS and confidence for continuation of standard regimen.

• Provides quick results for mixed or contaminated cultures, or negative cultures if smear-positive sediments are available.

• Provides quick confirmation of DR found by CDST.
  – Rule in DR, if mutations detected.
CDST—we still need it!

- CDST results, if correct, overrides MDST results
  - Sensitivity of MDST is not 100%
    - MDST detected no mutations, CDST can still be R.
      - Need to verify CDST results are correct—if in doubt, re-test making sure culture is pure & DST is properly performed.
  - Specificity of MDST is not 100%
    - MDST detected mutations but CDST can be S.
    - Need to recognize those mutations—silent mutations & others
      - Some MDST methods do not have this capability.
  - Association of some mutations with DR is not certain.
    - Need to rely on CDST. [more to discuss]
MDST methods

- **Probe-based**
  - Molecular beacons (GeneXpert)
    - Xpert got FDA-approval last month!
  - Line-probes (HAIN & LIPA)

- **SQ-based**
  - Sanger sequencing
  - Pyrosequencing
What to expect from various MDST methods

- **Molecular beacons**—
  - Mutation present or absent.

- **Line-probes**—
  - Few bands with specific mutations.
  - Presence or absence of wildtype bands.

- **SQ methods**—
  - Provide SQ.
  - Allow to identify specific mutations—known & new.
GeneXpert (GX)

- For identification of MTB & detection of RIF-R
- Simple, fast—hand-on time: few minutes.
- Testing raw specimens in 2 hr.
- Performance—
  - Identification of MTBC—
    - Sensitivity: Smear +: 98.2%; Smear-: 72.5%
    - Specificity: 99.2%
  - Detection of RIF-R
    - Sensitivity: 97.6%
    - Specificity: 98.1%
- Be aware: there are limitations!
Detection of Mutations with a Molecular Beacon
(Loop portion containing wildtype SQ)

Mutant Sequence

Wildtype Sequence

Amplicon

Loop

Quencher

Fluorophore

No mutations

Mutation present

Courtesy of Dr. Probert
Line Probe Assays

• Amplification of the target
  – Traditional PCR; one primer is biotinylated.

• Reverse hybridization
  – ssDNA-biotin hybridize to probes on membrane, if complementary.
  – Colorimetric detection
    • Observe presence or absence of bands.
Detect presence or absence of mutations & a few specific mutations.
MTBDRsL by HAIN

Conjugate ctrl
Amplification ctrl

gyrA locus ctrl
gyrA wild-type bands
gyrA mutations

rrs locus ctrl
rrs wild-type bands
rrs mutations

embB locus ctrl
embB wild-type
embB mutations

Colored marker

MTBC
90gtg
91ccg
94gcc
94aac, tac
94ggc
94cac
1401 G
1484 T
306ata
306gtg

XDR
Wildtype
Pre-XDR

MTBC
90gtg
91ccg
94gcc
94aac, tac
94ggc
94cac
1401 G
1484 T
306ata
306gtg

XDR
Wildtype
Pre-XDR

Conjugate ctrl
Amplification ctrl

gyrA locus ctrl
gyrA wild-type bands
gyrA mutations

rrs locus ctrl
rrs wild-type bands
rrs mutations

embB locus ctrl
embB wild-type
embB mutations

Colored marker
Probe-based MDST

- Detect presence or absence of a mutation(s).
- Do not inform what the mutation is
  - Assume all mutations are associated with DR.
  - Often true, but not always.
- When RIF-R rate is 2%, if the specificity is 96%, the PPV is 33%. [CA data]
- Due to low PPV, when a mutation is detected, confirmation by a sequence-based method is recommended.
GeneXpert

- No mutations detected in $rpoB$
  - Predict RIF-S, but unable to predict for INH
  - If INH-R is suspected, use other MDST methods
    - HAIN, PSQ or Sanger
- Mutations detected in $rpoB$
  - If RIF- R is not suspected, confirmation by a SQ-based MDST is recommended.
  - If RIF- R is suspected, confirmation by a SQ-based MDST is optional.
    - Probe E—most common, likely be true +
Sequence-based MDST

• Sanger sequencing
  • Gold standard

• Pyrosequencing (PSQ)
  – Realtime sequencing
Pyrosequencing workflow

PCR
Amplify target. One primer is biotinylated.

ssDNA prep
Capture ssDNA with streptavidin-coated beads. SQ primer anneals to DNA.

PSQ
Realtime sequencing. SQ data generated from the 1st base next to SQ primer.
Below are steps occur in pyrosequencer:

1. Incorporation of dNTP generates ppi.

2. APS + ppi → ATP, catalyzed by ATP sulfurylase.

3. ATP drives Luciferin → oxyluciferin, catalyzed by luciferase.

4. Light generated, proportional to dNTP incorporated, recoded by CCD.

5. Apyrase degrades unincorporated dNTP & ATP. When degradation is complete, another dNTP will be added.

6. Pyrogram shows sequential event of dNTP incorporated. The peak level is proportional to dNTPs incorporated.
SQ-based MDST

• Transparency with SQ provided
  – Enable to differentiate silent mutations from others.
  – Prevent reporting false-R.

• New mutations can be detected.
  – Defer interpretation to CDST.

• Emerging resistance can be recognized to certain extent (5:5 or 4:6, etc.).

• Allow to study MIC for each mutation
  – This knowledge is accumulable for future use.
SQ-based MDST

- No mutations detected –predicts DS.
- Mutations detected
  - For known mutations, based on past experience to interpret.
    - All mutations are not equivalent, some may not confer DR.
    - It is advisable to study MIC for each mutation.
  - Silent mutations are not associated with DR.
  - If the association of a mutation with DR is uncertain, defer interpretation to CDST.
  - For new mutations, defer interpretation to CDST.
Recommendations--CDST

• Two scenarios:
  – If DR detected, but not anticipated
  – Discordant CDST results with different methods or same method.

• Performing MDST can be helpful
  – If mutation detected, confirms DR results.
  – If mutation not detected, lean toward DS, repeat CDST.
    • Make sure the culture is pure
    • DST is performed properly
      – Drug solution is prepared and added properly
      – Inoculum is prepared properly.
Discordant CDST results: hard to resolve

- Sometimes even MDST cannot help!
  - Association of some mutations with DR is uncertain!
    - \textit{embB} mutations—may not confer EMB-R
    - \textit{rrs} 1401A/G—msy not confer CAP-R
    - Association of many \textit{pncA} mutations with PZA-R is still unknown.

- Problems with test (critical) concentrations?
- Problems with CDST methods?
Discordant CDST results “Flip-floppers”

- Poor reproducibility of CDST may occur when:
  - Some mutations cause MIC elevated slightly but lower than or close to the critical concentration.
  - Some strains with wildtype SQ (or have a mutation somewhere we do not know), but MIC of a drug is close to the critical concentration.
Flip-floppers (examples)

• rpoB 533 CCG & 511CCG
  – Borderline S or low level R.
  – RIF MIC = 0.5 ug/ml by MGIT
    • MGIT: likely to be S.
  – AP: S or R (low % R)
• rrs 1402C/T—AMK-R or S
  – Borderline S or low level R.
Flip-floppers = Borderline??

- TB DST does not have borderline category
  - Exception: PZA testing by BACTEC 460.
- For isolates with flip-flopping results
  - Lab may repeat and repeat, then report R or S based on 2/3 or 3/5 winners.
  - Clinicians takes S or R without knowing the flip-flopping nature of the result.
- Is it advisable to consider “borderline” for those flip-floppers?
  - When arsenals fall short for difficult TB cases, those flip-floppers may still be useful?
• A lot more to research.
• Microbes are smart!
• But, we do not give up!!
Thank You!

Comments & Questions!