

# 9th National Conference on the Laboratory Aspects of Tuberculosis



# Expected discrepancies between molecular and growth-based DST: Which technology is giving the right answer?

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# Disclosure

Dr. Desmond reports no conflicts of interest.

Opinions expressed are those of the speaker alone and do not represent official positions of any institution.

# Drug susceptibility testing in USA

Systems for drug susceptibility testing (DST) in the USA were developed and standardized in the 1960's and '70s.

DST is considered generally reliable, and treatment regimens guided by DST are generally successful.

Because culture-based DST is considered generally reliable, it has been used as a reference point to assess the accuracy of newer molecular methods.

BUT....

# There are many obstacles to accurate and reproducible DST results by culture or molecular methods...

- New culture-based DST methods are calibrated to match results of existing methods, but the calibration is not very precise
  - Which drug concentration in a serial 2 fold dilution scheme will give results closest to the reference method?
- Culture media contain undefined biological components such as pancreatic digest of casein or bovine albumin
  - These can vary in composition from batch to batch
- **Culture-based DST is not a perfect system**

# Expected discrepancies (1)

**Between culture-based methods:** agar proportion (AP) vs. rapid broth (MGIT or VersaTrek)

Example A:

**Ethambutol:** November 2010 MPEP– duplicate isolates with Met306Val mutation

- 90% of labs using AP detected drug ®
- 23% of labs using MGIT detected drug ®

Note: for most cultures, ethambutol results by AP and MGIT agree. The frequency of discrepancies is unknown. But there is a tendency for AP to detect more resistance than MGIT

# Expected discrepancies (1)

Between culture-based methods: AP vs. rapid broth  
(MGIT or VersaTrek)

Example B, from Expanded MPEP: Rifampin

Culture No.	<i>rpoB</i> mutation	% resistant to rif by agar proportion	% resistant to rif by MGIT
EM009	Asp516Tyr	40%	0%
EM011	Duplicate of EM009	60%	0%
EM026	Leu511Pro	20%	0%

Again, frequency of discrepancies between AP and MGIT is unknown, but there is a tendency for AP to detect more RIF resistance than MGIT.

# Expected discrepancies (2)

**Between molecular methods (probes vs. sequencing)**

**Silent or synonymous mutations** in the rifampin-resistance determining region (RRDR) of *rpoB* gene:

**Cepheid GeneXpert:** mutation detected, likely rifampin resistant

**DNA sequencing:** silent or synonymous mutation detected, likely drug susceptible



# Expected discrepancies (3)

Between molecular and **culture-based** drug susceptibility testing methods

**Heteroresistance:** presence of both susceptible and resistant TB bacilli in a specimen

- Culture-based DST is designed to detect resistant bacilli if they make up  $>1\%$  of the population
- Probe-based assays like molecular beacons will detect presence of wild-type sequence and predict susceptible
- Sequencing-based method likely to miss the presence of mutated, resistant bacilli unless they make up  $>30\%$  of the population

# Expected discrepancies (3)

**Between molecular and culture-based** drug susceptibility testing methods, cont'd

“Disputed” or “low level resistance” mutations in *rpoB*, e.g. Asp516Tyr or Leu511Pro

- GeneXpert: mutation present, resistance predicted
- Sequencing based method: exact mutation identified, clinical significance beginning to be understood
- Culture-based DST: may test as susceptible or resistant (AP more likely to detect resistance than MGIT)

# Expected discrepancies (3)

Between molecular and culture-based drug susceptibility testing methods, cont'd

## **Silent (synonymous) mutations:**

- GeneXpert: mutation present, likely resistance
- DNA sequencing: silent mutation, likely susceptible
- Culture-based DST: susceptible

# Which technology gives most accurate results? It depends:

	Heteroresistance (mixture of S and R)	Silent mutation	Low level RIF resistance
Culture- based DST	X	X	
Probe-based molecular (e.g. GXP)			X
DNA sequencing		X	X

# How frequently do these challenges to the accuracy of DST occur?

- **Heteroresistance:** unknown. Should be more common for patients treated briefly than for patients who have undergone longer treatment regimens
- **Silent mutations in *rpoB* gene:** their frequency as a % of total *rpoB* mutations depends on the frequency of rifampin resistance. Lower in countries with high burden of MDRTB. In USA, silent mutations in *rpoB* may approach 1/5 of all *rpoB* mutations.
- **“Low level resistance” mutations in *rpoB*** which can cause discrepant results in MGIT: 11 to 13% of all *rpoB* mutations in Congo and Bangladesh (van Deun JCM 2013 51:2633), but may be different in the USA

# What is the “gold standard” method?

- *Agar proportion?* Too slow, may miss low-level rifampin resistance, can't be used for PZA
- *GeneXpert MTB/RIF?* May mis-call silent mutations and may miss heteroresistance
- *MGIT?* May miss some resistance to rifampin and ethambutol
- *DNA sequencing?*
  - May miss heteroresistance
  - Not all mutations associated with resistance are known

# If your gold standard is tarnished, what have you got?



- Research questions
- Algorithms

**Decontaminated, concentrated**

**sputum sediment**

**GeneXpert MTB/Rif**

**Acid-fast smear**

**Culture  
(DST if**

**Retained  
sediment**

**TB pos**

**TB pos**

**TB**

**positive)**

**Rif suscept.**

**rpoB mutation**

**negative**

**Send retained**

**Sediment for**

**DNA sequencing**

**Detect silent and low level  
resistance mutations**

**Detect**

**heteroresistance**





# Ethambutol: can research lead us out of the fog?

# Broth drug susceptibility testing for ethambutol (EMB)

- Radiometric BACTEC 460/12B medium: EMB test concentration 2.5 ug/ml
  - Test concentration may have been too low
  - Some resistant results were not confirmed by agar proportion
- MGIT medium: EMB test concentration 5 ug/ml
  - Some false susceptible results occur, in strains with *embB* mutations, which test resistant by agar proportion (AP) method

## Culture-based ethambutol DST for TB strains with wild-type *embB* sequence (expanded MPEP)

Strain	<i>embB</i> sequence	AP result Lab A	AP result Lab B	MGIT MIC Lab C
2	WT	R	R	>5 (R)
3	WT	R	R	>5 (R)
6	WT	S	S	≤3 (S)
7	WT	R	S	4 (S)
8	WT	S	S	≤3 (S)
10	WT	R	R	>5 (R)
16	WT	R	S	4 (S)
21	WT	S	S	≤3 (S)
27	WT	S	S	≤3 (S)

**Some ethambutol resistance is not detected by *embB* sequencing.**

# Culture-based ethambutol DST for TB strains with met 306 ile mutation in *embB* gene

Strain	<i>embB</i> mutation	AP result lab A	AP result lab B	MGIT MIC lab C
4	met 306 ile	R	S	5 (S)
13	met 306 ile	R	S	>5 (R)
15	met 306 ile	R	*	4 (S)
22	met 306 ile	R	R	>5 (R)
23	met 306 ile	S	*	4 (S)
28	met 306 ile	R	S	>5 (R)
29	met 306 ile	R	S	≤3 (S)
30	met 306 ile	R	*	≤3 (S)

Isolates with the same mutation may differ widely in suscept. vs. resistance.

Labs with good QC, doing AP DST for EMB may differ significantly in results.

# Findings from expanded MPEP regarding ethambutol

- Some strains resistant to ethambutol do not have mutations in the *embB* region which is routinely sequenced
- Laboratories with strong quality control programs may differ from each other in their ethambutol results with some strains
- TB strains with the same *embB* mutation may differ significantly in their ethambutol MICs

# Research questions re: EMB testing in MGIT

Where is the **clinical correlation**? We can work to make different lab methods agree, but are we really doing a better job of predicting treatment success or failure?

How a **rodent study** might help:

- Develop a set of strains with a range of EMB MICs
- Infect rodents with these strains, then treat with EMB doses equivalent to human therapy
- Sacrifice mice following treatment and evaluate whether EMB had been effective in inhibiting growth in lungs
- Establish an MIC cut point, above which EMB treatment is not likely to be effective

# Ethambutol research questions

- TB strains with wildtype sequences in *embB* may have high EMB MICs: what other genes and mutations are associated with resistance?
- TB strains with the same *embB* mutation may have significantly different MICs: how do combinations of mutations affect susceptibility or resistance quantitatively?
- Linking of whole genome sequencing data with MIC values may provide answers

# TB strains which are ethambutol susceptible by MGIT, resistant by agar proportion method

- May indicate diminished activity of ethambutol, but the drug may still contribute to treatment regimens
- Clinical relevance not known at present



# Does variability in culture-based DST systems make them less reliable? The example of PZA

Supplement lot number	PZA drug lot number	Medium lot number	MIC for H37Rv	Date
4262976	4262938	4262838	100	April 2015
4038491	4066064	3305456	50	August 2014
3101100	3143405	3305456	25	January 2014

Quality control MIC testing of stock strain with new batch of PZA drug and supplement

# Lot to lot variability in culture media sometimes affects drug susceptibility testing results

Guthertz, L., et al. 1988 JCM 26:2338: “This study demonstrates that individual lots of components of this basal medium may vary significantly in their suitability for susceptibility testing, and failure to detect such variation may dramatically affect susceptibility profiles.”

- Medium base and (OADC) supplement contain biological materials which may vary from lot to lot.
- This may affect growth support or drug activity in media made with these components

# Maintaining lot to lot consistency of DST media and components

- One approach: define medium components as carefully as possible, and do pre-market testing of components to assure lot to lot consistency
  - A manufacturer's responsibility—will they do it?
- Another approach: when a new batch of medium component is received, make up some media with new and some with old component and compare growth support and drug MICs between new and old lots
  - DST laboratory's responsibility (an onerous one)

# Consistency over time: culture-based DST vs. DNA sequencing

- Culture-based DST may yield inconsistent results due to changes in medium composition which are very difficult to avoid
- DNA sequences are objective and not subject to subtle changes in manufactured components
- But first the links between DNA sequences and *in vitro* and *in vivo* drug resistance must be established
  - It may take decades of work to establish the links between mutations and combinations of mutations on drug resistance

# Pyrazinamide

Chedore, et al. 2010. JCM 48:300: of 57 isolates which tested PZA resistant in MGIT, only 33 (58%) were resistant on repeat testing.

Zhang, et al. 2014: Current phenotype-based susceptibility testing is not reliable due to false resistance; sequencing of the *pncA* gene represents a more rapid, cost-effective, and reliable molecular test for PZA susceptibility testing... (Microbiol. Spectrum 24(4).)

# Take home points

- DNA sequencing provides rapid results not altered by subtle changes in reagents
  - Correlation between sequence changes and drug susceptibility may be complex and multifactorial for some drugs. Elucidation may require extensive study.
  - Some mutations are clearly associated with resistance.
- Culture-based DST may be affected by subtle changes in culture medium ingredients
  - Is rigorous QA by the end user practical?
  - Would more rigorous pre-market testing by manufacturer help?
- For ethambutol, clinical correlation of DST may need to be re-established.
- Testing algorithms may be designed to overcome weaknesses in current test methods.

# Thank you



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