Disclosures

• None
Objectives:

• Describe laboratory methods used for the detection, identification, genotyping, and drug susceptibility testing of *M. tuberculosis*
Overview

- Introduction to Mycobacteria
- Stains for Mycobacteria
- Culture of Mycobacteria
- Molecular methods for identification of *M. tuberculosis*
  - from culture isolates
  - directly from specimen
- *M. tuberculosis* drug resistance testing
  - rapid broth-based methods
  - molecular markers of resistance
Mycobacteria - General Characteristics

- aerobic, rod-shaped bacilli
- often found in the environment (soil, water)
  - an exception is *M. tuberculosis*
- hardy organisms - many species, including *M. tuberculosis*, are resistant to drying and most disinfectants
The Mycobacterial Cell Wall is Unique

- contains >60% lipid
  - very long chain (C$_{60}$-C$_{90}$) fatty acids called mycolic acids
  - waxes
- contrast with:
  - other Gram positive organisms contain ~5% lipid
  - Gram negative organism contains ~20% lipid
- mycolic acids make the cell surface extremely hydrophobic and resistant to staining with basic aniline dyes or penetration by drugs
Mycobacteria
180 recognized species and subspecies

- **M. tuberculosis** complex
  - includes:
    - *M. tuberculosis*
    - *M. bovis* (cattle, unpasteurized milk)
    - *M. bovis* BCG (vaccine strain)
    - other species in the complex: *M. africanum, M. microti, M. canetti, M. bovis* subsp. *caprae, M. pinnepedi, M. mungi*

- Non-tuberculosis mycobacteria (NTM’s, aka MOTTs):
  - slowly growing (> 7 days to appear on solid media)
    - eg., *M. avium, M. marinum, M. kansasii*
  - rapidly growing (growth within 7 days on solid media)
    - eg., *M. fortuitum, M. chelonae, M. abscessus*
  - uncultivatable in the lab - *M. leprae*
What Happens to the Patient’s Specimen When It is Sent to the Lab?

Specimen

Perform Acid-Fast Smear – Mycobacteria Present?

Smear

Perform culture on specialized medium

Culture

If culture grows, identify mycobacterium using molecular methods (hybridization probes, MALDI-TOF MS, or DNA sequencing)

ID

Perform drug resistance testing on isolate

AST

PCR

Perform Direct PCR for *M. tuberculosis*
Specimen types

- virtually any specimen type can be accepted for smear and culture
- most common is sputum
  - if possible, obtain 3 sputum specimens for AFB smear and culture
  - in children who have difficulty producing sputum, gastric wash is an alternative
  - first morning specimens preferred
  - on 3 separate days if possible
Preparation of smears

Non-sterile specimens (e.g., sputum) often contain bacteria which outpace/overgrow any mycobacteria present

Non-sterile specimen processing
• N-acetyl-cysteine (mucolytic agent)
• 2% NaOH (decontaminating agent)
• NALC-NaOH treatment helps liberate mycobacteria and reduce amount of other bacteria present
• pellet
• rehydrate /w sterile PBS
• prepare and examine smear
Stains for mycobacteria

• Why perform a stain?
  • Rapid, inexpensive indication of whether the specimen contains mycobacteria
  • A stain may take about an hour to perform and report
  • A mycobacterial culture requires days to weeks
  • Molecular methods such as PCR are also quick but cost more and we only have good ones for Mtb

• What kind of stain is done?
  • Mycobacteria do not stain with the Gram stain
  • An “acid-fast” stain is used (eg., auramine/rhodamine, Ziehl-Neelsen, or Kinyoun stain)
Mycobacterium tuberculosis does not stain well with the Gram stain.

*M. tuberculosis* “ghosting” on Gram stain
Acid-fast stains for mycobacteria

- mycobacteria are referred to as “acid-fast” bacilli (AFB)
- a complex is formed between mycolic acid and dye used in the stain (e.g., carbol-fuchsin or auramine O)
- this complex is resistant to destaining by mineral acids (this is why mycobacteria are called “acid-fast”)
- so mycobacteria retain the carbol-fuchsin or auramine O stain and other bacteria do not
### Ziehl-Neelsen Stain

Kinyoun Modification

<table>
<thead>
<tr>
<th>Acid Fast Organisms</th>
<th>Not Acid Fast Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Organsms" /></td>
<td><img src="image2" alt="Organsms" /></td>
</tr>
</tbody>
</table>

- A small amount of organism suspended in saline solution is fixed on a slide.
- Slide is flooded with Carbol Fuchsin and phenol for 3 minutes, and gently rinsed with water.
- Slide is decolorized with 3% HCl in 70% alcohol until color appears to be removed (approx. 2 mins), and rinsed with water.
- Slide is flooded with methylene blue counterstain for 30 secs, rinsed with water and air dried.

http://www.ratsteachmicro.com/Mycobacteria_notes/
Ziehl-Neelsen stain
uses heat to help drive fuchsin stain into waxy cell wall; phenol as mordant to fix stain;
(Kinyoun stain method – no heat, instead uses higher concentration of phenol and
fuchsin dye to aid uptake; less effective as direct stain)

AFB’s stain in red; non-AFB’s stain in blue
Auramine-rhodamine stain
Acid-fast stains - Issues

- Acid-fast stains are not very specific
  - indicates whether a mycobacterium is present in the specimen
  - does not allow us to know which mycobacteria it is
    - *M. tuberculosis* looks like all the other mycobacterial species on an acid-fast stain
- Acid-fast stains are not very sensitive
  - need 1000-10,000 CFU/ml for a positive AFB smear
Tuberculosis Cases with Pulmonary Involvement by Initial Sputum AFB Smear Result, Minnesota, 2010-2014

- Positive: 37%
- Negative: 50%
- Not done*: 13%

*53% of pulmonary cases without sputum smear results were under 15 years of age

N = 460
# 2-3 AFB Smears are More Sensitive than 1 Smear

## Yield of Serial AFB Smears

<table>
<thead>
<tr>
<th>Study</th>
<th>% of Total Positives Detected by:</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; Smear</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; Smear</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; Smear</td>
<td></td>
</tr>
<tr>
<td>Walker et al. (2000), <em>Int J Tuberc Lung Dis</em>, 4:246.</td>
<td>77.1%</td>
<td>15.0%</td>
<td>7.9%</td>
<td></td>
</tr>
<tr>
<td>Ipuge et al. (1996), <em>Trans R Soc Trop Med Hyg</em>, 90:258.</td>
<td>83.4%</td>
<td>12.2%</td>
<td>4.4%</td>
<td></td>
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<tr>
<td>Mathew et al. (2002) <em>J Clin Microbiol</em>, 40:3482-4 (low prevalence pop.)</td>
<td>89.4%</td>
<td>5.3%</td>
<td>5.3%</td>
<td></td>
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</tbody>
</table>
Acid-Fast Smears Prepared from Early Morning Sputum Specimens Have Better Sensitivity

<table>
<thead>
<tr>
<th>Study</th>
<th>Spot (Random) Specimen Positive (%)</th>
<th>Early Morning Specimen Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abraham et al, 2012, <em>Indian J Med Res</em>, 135: 249-51 (smear is positive)</td>
<td>21/49 (43%)</td>
<td>32/49 (65%)</td>
</tr>
</tbody>
</table>
Mycobacteria Cultures

Necessary to obtain an isolate of the mycobacterium for:

- species identification
- antimicrobial susceptibility testing
Laboratory Identification of Mycobacteria

Safety Considerations:
M. tuberculosis/M. bovis are BSL III Pathogens
Culture of *M. tuberculosis* complex

- Sensitivity of culture is much better than smear
  - a positive AF smear requires 1000-10,000 CFU/ml of specimen
  - a positive mycobacteria culture requires only 10-100 CFU/mL of specimen

**Culture**

- 2 types of media used:
  - Solid Medium (Lowenstein-Jensen (LJ) or Middlebrook)
  - Broth (Liquid) Medium (FDA-cleared systems - Bactec MGIT and Trek VersaTREK)
  - In general, mycobacteria grow faster in broth but there are some strains that grow better on solid medium
M. tuberculosis Colony Morphology on Solid Medium

Note the “rough and buff” morphology typical of M. tuberculosis
BACTEC MGIT 960 Culture System

MGIT - Mycobacterial Growth Indicator Tubes (Becton Dickinson)
- fluorescent indicator in bottom of tube quenched by $O_2$
- $\uparrow$ mycobacterial growth = $\downarrow O_2$ and $\uparrow$ fluorescence
VersaTREK System

- mycobacterial growth causes changes in bottle headspace pressure which are detected by the instrument; sponges in bottle provide increased surface area for growth

Tuberculosis Cases by Mycobacterial Culture Result, Minnesota, 2010-2014

- Positive for *M. tbc* 76%
- Negative 20%
- Not done 4%

N = 732
Identification of *M. tuberculosis* complex from culture
Identification of MTB from Culture Isolates: Nucleic Acid Hybridization Probes

- **Use for Isolates Grown in Culture**
  - no DNA amplification step so need lots of target nucleic acid
  - add probe with unique, complementary sequence to known species; chemiluminescent detection
  - identification within 2-3 hours after growth in culture

If the culture isolate is MTB, the DNA probe will bind isolate’s rRNA and produce a signal.
Identification of MTB from Culture Isolates: Nucleic Acid Hybridization Probes

- Hologic Gen-Probe AccuProbes® (nucleic acid hybridization probes) available for:
  - *M. tuberculosis* complex
  - *M. avium* complex
  - *M. gordonae*
  - *M. kansasii*

- FDA-approved for identification of culture isolates

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> complex</td>
<td>99.2%</td>
<td>99.0%</td>
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</tbody>
</table>
Identification of MTB from Culture Isolates: Line Probe Hybridization Assays (Hain Lifesciences or Innogenetics)

- Genus- and species-specific probes bound to nitrocellulose membrane
- DNA from lysed culture isolate hybridizes to the probe for identification.
- Hain products (as an example):
  - GenoType Mycobacterium CM and AS
    - *M. tuberculosis* complex and 29 nontuberculous mycobacteria on 2 strips
  - GenoType MTBC
    - Differentiation of *M. tuberculosis* complex
  - GenoType MTBDR plus
    - *M. tuberculosis* complex plus wt and mutant *rpoB, katG, inhA*

Not approved for diagnostic use in U.S. at this time
Source: http://www.hain-lifescience.de
Identification of MTB from Culture Isolates: DNA Sequencing

- Sanger dideoxy sequencing is the current gold standard for mycobacteria identification
  - Various targets are useful (\textit{rpoB}, \textit{hsp65}, 16S rDNA gene, etc.)
  - uses broad range primers that will amplify all mycobacteria species
  - hypervariable region between primers used to distinguish species

Identification of MTB from Culture Isolates: DNA Sequencing

TAT is next day after growth of isolate in culture:

- Lyse Isolate (safety, expose DNA)
- Amplify DNA & incorporate ddNTPs
- Run Capillary Electrophoresis to separate DNA fragments based on size; read sequence
- Match sequence to Mycobacterial sequencing library entries to ID species
Identification of MTB from Culture Isolates: MALDI-TOF Mass Spectrometry

- Matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry (MS)
- changing the way we identify microbes in microbiology laboratories
- heavily utilized in many laboratories for bacterial and yeast identification
- we also use MALDI-TOF MS as the method of choice for identification of mycobacteria
Identification of MTB from Culture Isolates: MALDI-TOF MS

10ul loop-ful of organism → Beads+500μl 70% Ethanol → Incubate room temp 10 min → Bead Beat 2 minutes

BSL3 Activities

10ul loop-ful of organism → Beads+500μl 70% Ethanol → Incubate room temp 10 min → Bead Beat 2 minutes

BSL2 Activities

Centrifuge 5 min → Speed Vac 10 min → 70% Formic Acid & Acetonitrile

Spot 1ul sample + 2ul of Matrix

MALDI-TOF

start to finish takes ~2 hrs for 24 samples

Mayo Clinic Center for Tuberculosis
What happens inside the Mass Spectrometer?

Compare patient's isolate spectrum to library of mycobacterial spectra to determine the species.
Advantages of MALDI-TOF MS for Mycobacteria Identification

• Advantages
  • Similar work-flow regardless of organism (bacteria, yeast, mycobacteria, mold)
  • Cost effective and “green” w/ low consumable costs
  • Rapid turn around time, high throughput, small footprint
  • Low exposure risk for lab staff
  • Adaptable – RUO versions allow databases expansion by user

• Limitations
  • Need pure isolate grown in culture (not a direct method)
  • Phase of growth, media, timing all factors
  • Databases need expansion for less common organisms
  • Instrument maintenance costs and downtime can be significant
  • Regulatory issues for RUO databases
What Happens to the Patient’s Specimen When It is Sent to the Lab?

- Specimen
  - PCR
  - Perform Direct PCR for *M. tuberculosis*

- Smear
  - Perform Acid-Fast Smear – Mycobacteria Present?

- Culture
  - Perform culture on specialized medium
  - If culture grows, identify mycobacterium using molecular methods (hybridization probes, MALDI-TOF MS, or DNA sequencing)

- ID

- AST
  - Perform drug resistance testing on isolate
Direct Identification of *M. tuberculosis* complex from patient specimen *without waiting* for growth in culture

In general, we have good molecular methods for direct detection of MTB but not for NTMs
Nucleic Acid Amplification-based (NAA) tests for MTB

• CDC recommendation:
  • NAA testing be performed on at least one (preferrably the first) respiratory specimen from each patient with suspected pulmonary TB
    • if it would alter case management
    • If it would alter TB control activities
  • NAA testing does not replace the need for culture
NAA Tests for Direct Detection of MTB

• FDA-cleared
  • Hologic/Gen-Probe MTD
  • Cepheid GeneXpert MTB/RIF
• CE-marked/RUO in U.S.
  • Hain LineProbe
• Laboratory Develop Tests (LDTs)
  • Rapid cycle/real-time PCR
Direct Detection of MTB from Patient Specimens

*Mycobacterium tuberculosis* Direct Test (MTD) (Hologic Gen-Probe)

- people frequently refer to this as the “TB probe” assay but that is not correct; this is a PCR-like amplification method
  - transcription-mediated amplification of *M. tuberculosis* complex rRNA directly from respiratory specimens
- clinical specificity: 99-100%
- clinical sensitivity:
  - smear positive: 91-95%
  - smear negative: 83-100%
- technically challenging test
  - inhibition from specimen components a concern;
  - open PCR system so false positives due to cross-contamination of specimens are possible.
  - cross-reactions occur w/ some rare mycobacteria: *M. celatum, M. terrae*-like organisms, *M. holsiaticum*
Direct Detection of MTB from Patient Specimens
Cepheid Xpert® MTB/RIF Test

- WHO-endorsed
- Runs on the Cepheid GeneXpert platform
- FDA-approved for respiratory specimens
- Detects *M. tuberculosis* complex and provides information about RIF resistance
- Results in about 2 hrs; minimal hands-on needed

Source: www.finddiagnostics.org
Xpert MTB/RIF accuracy for detection of *Mtb* complex

- Limit of Detection is 131 CFU/ml (package insert)

- **Chang et al, 2012, J Infect 64:580-8:**
  - Meta-analysis of 18 studies with 10,224 patients total
  - Pulmonary TB:
    - Sensitivity, Smear positive disease – 98.7%
    - Sensitivity, Smear negative disease – 75.0%
    - Specificity - 98.2%
  - Extrapulmonary TB:
    - Sensitivity - smear positive, 95.2%; smear negative 70.7%
    - Specificity – 82.6%

- **Time to diagnosis comparison:**
  - Smear microscopy = same day (but non-specific)
  - Broth culture took an average of 16 days
  - Solid media plate cultures took an average of 20 days
  - Xpert MTB/RIF– same day diagnosis
Xpert MTB/RIF as an alternative to AFB smear

• 2015, FDA expanded intended use of Xpert MTB/RIF Assay to include testing of either one or two sputum specimens as an alternative to examination of serial acid-fast stained sputum smears to aid in the decision to d/c airborne isolation for patients with suspected pulmonary tuberculosis.

• 1-2 negative Xpert MTB/RIF sputum assay results reported to be as good as 2-3 negative acid-fast sputum smears

• the decision to perform 1-2 MTB/RIF assays in determining the need for continued isolation should be based on specific clinical circumstances and institutional guidelines.
Direct Detection of MTB from Patient Specimens
Line Probe Assays
(Hain Lifesciences)

**M. tuberculosis** complex direct detection

Not approved for diagnostic use in the U.S.

Source: [http://www.hain-lifescience.de](http://www.hain-lifescience.de)
Direct Detection of MTB from Patient Specimens
Laboratory-developed PCR Tests (LDTs)
Example of Real-time PCR Workflow in our Laboratory

specimen or culture lysis, inactivation and processing

DNA extraction

PCR amplification and detection

Approximate turn-around time = 4h
Direct Detection of MTB from Patient Specimens
Laboratory-developed PCR Tests (LDTs)

Advantages

- closed PCR system – reduced opportunity for false-positives
- good sensitivity and specificity but it can vary since each test developed/verified independently
- often less expensive
- some can be used on a wider variety of specimen types included smear negative specimens and formalin-fixed, paraffin-embedded tissue blocks

Limitations

- Often not as well-characterized as FDA-cleared tests
  - How does sensitivity and specificity compare to cleared tests?
  - Payer reimbursement and regulatory issues for LDTs
General Limitations of NAA tests for Direct Detection of *M. tuberculosis*

- Inhibition from specimen components a concern for falsely negative results
  - Inhibition control needed unless system lab has checked for inhibitors in all specimen types
- PCR detects presence of nucleic acid but doesn’t indicate if the organism is still viable
  - patient could be being treated successfully but may still have a positive PCR result
- Culture is more sensitive so always perform culture too
  - negative PCR result does not rule out *M. tuberculosis* infection
  - culture isolate is needed for drug susceptibility testing
Drug Resistance Testing of *M. tuberculosis* complex
**M. tuberculosis** complex Drug Resistance Testing

- agar proportion is the current gold standard for all drugs except pyrazinamide
  - not rapid (14-21 days)
  - labor-intensive, technically complex
  - no FDA-cleared, commercially-available kit

Organism is resistant to drug A in the upper right compartment (>1% of inoculum shown by upper left control quadrant is growing in presence of drug). Organism is susceptible to drugs B & C in the lower compartments. Control quadrant in upper left contains no drugs.
Rapid Broth Susceptibility Testing for MTB

FDA-cleared, semi-automated with MGIT or VersaTREK systems

Compare growth rates in bottles/tubes +/- critical concentrations of drug

CDC goal is results for first-line drugs reported within 15-30 days after receipt of the specimen
**M. tuberculosis** complex resistant isolates

- If the isolate is resistant to any agent
  - preliminary report issued
  - consider confirming resistance by 2nd method or 2nd lab
  - consider initiating testing of secondary agents to avoid delays

- If the isolate is resistant to only PZA consider
  - speciation
    - *M. bovis* is mono-PZA-resistant
    - most isolates of *M. tuberculosis* are PZA-susceptible
Newest Method for Mtb DST LDT (Not FDA-cleared) MIC Plate

- broth microdilution method
- multi-center studies supporting FDA-submission completed
- rapid (14 days)
- contains INH, RIF, EMB and 9 second-line drugs
- test 1st and 2nd line drugs simultaneously with same inoculum
- provides MIC endpoint – helpful for isolates with MIC near critical concentration (CC) breakpoint that give fluctuating results w/CC method

Molecular detection of *Mtb* drug resistance markers

Why?

Rapid determination of potential drug resistance compared with phenotypic methods

Limited availability at this time except for the CDC MDR TB program
LDT PCR at Mayo
Direct Detection of INH Resistance Mediated through KatG S315T Mutation

- primary use of this PCR is detection of MTB
- it can give some direct information about INH resistance.
- there are other mutations associated with INH-R so a WT pattern does not guarantee INH susceptibility
- possible INH-R reported if S315T mutation is seen
Pyrazinamide resistance – Sequencing of \( pncA \)

- Broth susceptibility testing of PZA can overcall resistance
  - MGIT (up to 68% false resistance)
    - Piersimoni C et al., 2013, J Clin Microbiol. 51:291-4
    - Simons SO et al., 2012, J Clin Microbiol. 50: 428-34
  - VersaTREK (~70% false resistance)
    - Simner PS et al., manuscript in preparation
- Sequencing of the \( pncA \) gene from culture isolates can help
  - mutations associated with resistance occur throughout this 558bp gene so sequence entire gene and promoter region
  - performed by CDC, Mayo or the NYS DOH Wadsworth Center
Xpert MTB/RIF and Rifampin resistance

• Target is $rpoB$: gene encoding beta subunit of bacterial RNA polymerase

• Mutations in an 81bp region of the $rpoB$ gene are responsible for ~96% of RIF resistance in $Mtb$;

• also predicts MDR TB since the majority of RIF-resistant isolates will also be INH-resistant

• Some false positive RIF resistance with Xpert
  • PPV is lower in low prevalence settings
  • CDC recommends reporting Xpert RIF-R as a preliminary result pending confirmation with sequencing; growth-base DST is still required
Molecular Detection of *M. tuberculosis* Drug Resistance at the CDC

- offered for *M. tuberculosis* complex isolates and nucleic-acid amplification-positive (NAAT+) sputum sediments
- perform pyrosequencing and conventional sequencing
- provides rapid identification of mutations associated with resistance to many TB drugs
- limitations include
  - insufficient data to definitively associate all mutations detected with resistance;
  - not all mechanisms of resistance are known
  - not all resistance loci are sequenced
- use in conjunction with conventional DST results
Molecular resistance testing for MTB at the CDC

<table>
<thead>
<tr>
<th>Drug</th>
<th>Locus/Loci examined</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>rifampin</td>
<td>rpoB</td>
<td>97.1</td>
<td>97.4</td>
</tr>
<tr>
<td>isoniazid</td>
<td>inhA &amp; katG</td>
<td>86.0</td>
<td>99.1</td>
</tr>
<tr>
<td>fluoroquinolones</td>
<td>gyrA</td>
<td>79.0</td>
<td>99.6</td>
</tr>
<tr>
<td>kanamycin</td>
<td>rrs &amp; eis</td>
<td>86.7</td>
<td>99.6</td>
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<tr>
<td>amikacin</td>
<td>rrs</td>
<td>90.0</td>
<td>98.4</td>
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<tr>
<td>capreomycin</td>
<td>rrs &amp; tlyA</td>
<td>55.2</td>
<td>91.0</td>
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<tr>
<td>ethambutol</td>
<td>embB</td>
<td>78.8</td>
<td>94.3</td>
</tr>
<tr>
<td>pyrazinamide</td>
<td>pncA</td>
<td>86.0</td>
<td>95.9</td>
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Summary

- AFB stains are rapid but insensitive and nonspecific.
- Mycobacterial culture should always be ordered together with AFB stain.
- Identification after growth in culture is rapid using molecular methods.
- Direct identification of MTB using molecular methods most often uses smear-positive respiratory specimens; certain methods allow for other specimens.
- Molecular detection of some drug resistance markers is available for Mtb culture isolates and directly for smear-positive respiratory specimens.
Questions & Discussion
Question #1
Which of the following statement is TRUE when ordering a mycobacterial smear

A. the Gram stain is good for detecting mycobacteria
B. acid-fast stains are insensitive
C. an acid-fast stain can be used to differentiate *M. tuberculosis* from nontuberculous mycobacteria
D. it’s OK to order an acid-fast smear without also ordering a mycobacteria culture to save money
Question #2
Which statement is TRUE about the GenXpert MTB/RIF PCR test?

A. the PCR test will also be positive for nontuberculous mycobacteria

B. the PCR test should always be negative if the patient’s treatment is working

C. if the MTB/RIF PCR test indicates RIF resistance, the *M. tuberculosis* isolate will always be resistant to RIF

D. 1-2 negative MTB/RIF PCR tests can be used to remove a patient from airborne isolation