Common Issues Observed in the International TB Laboratories:
What should laboratories do to improve?

June 2010
Peggy Coulter MDE, MT (HEW)
Senior International QA/QC Coordinator
Patient Safety Monitoring in International Laboratories (SMILE)
mcoulte1@jhmi.edu

Facilities

• The CDC recommends that work involving manipulations of TB cultures such as DST be done in a BSL-3 laboratory. However, this work may be done in a BSL-2 laboratory providing the exhaust air from the laboratory is discharged to the outdoors, the ventilation is balanced to provide directional airflow into the room, access to the room is restricted when work is in progress, and the recommended practices and equipment for BSL-3 are followed while handling specimens and mycobacterial cultures.
• Post warning signs- Limit access - Lock doors
• Keep windows closed during testing
• Remove cloth chairs
• Provide sinks locally, soap and clean towels
• Connect all equipment to back-up power sources
• Post emergency spill procedures in lab

Monitoring of Quality Indicators

• Specimen Collection and Transport – review instructions for collection and transport/storage temperatures; complete labeling of containers
• QC Records – documentation, corrective actions, supervisor review
• EQA – acceptable procedures and performance; perform and document investigation steps; do not split EQA specimens or treated differently from patient specimens
• Monitor contamination rates: A rate less than 3% suggests overly harsh decontamination; 3-5% is ideal, 5-8% is acceptable.
• Maintain documents for traceability of all test procedure batches
  — Record the lot numbers for all reagents.
  — Keep a record of the batch of specimens processed at one time, date of inoculation, person who did the work, time-to-positivity, smear results from positive tube, contamination, etc.
Monitoring of Quality Indicators

- Every 3 – 6 months calculate the following:
  - Smear Positive, Culture Positive – Total #, Average Time to Detection
  - Smear Negative, Culture Positive – Total #, average Time to Detection
  - Smear Positive, Culture Negative – Total #
  - Smear Negative, Culture Negative - Total #
  - Positive Control – Average Time to Detection
  - Contamination – Total #, Average Time to Detection
- An abrupt shift in any category of specimen data would indicate some change in the laboratory practices or reagents.
- All changes or variances to standard practices or manufacturer procedures must be validated
- Have a second person look at a selected number of positive and negative smears and those that have very few AFB on the smear. Compare results of the two technicians.

Staffing

- Provide adequate staffing for testing volumes and extent of procedures
- Staff Training/Competency assessment for all procedures
- Continuing Education
- Develop clear, detailed written SOPs for all procedures (Preanalytical, analytical, post analytical); director approved with yearly review; available on bench
- Posters and work instructions with director approval and annual review

Supplies

- Disposables versus Washing: monitor for correct use
- Replace wash brushes as needed
- Provide clean water for adequate rinsing steps
- Provide training for washroom procedures
- Provide PPE (sizes and availability)
- Storage of reagents/supplies
- Water qualities testing of both distilled and tap water (if used for staining procedures)
**Equipment - Centrifuges:**

- Use correct speed in g not rpm
  - 3,000 x g for 15 minutes will sediment 95% of mycobacteria in a digested sputum specimen. The specific gravity as tubercle bacilli ranges from 1.07 to .79 making centrifugal concentration of specimens ineffective if the RCF is not 3000 x g. Mycobacteria being hydrophobic are hard to centrifuge down. Lower centrifugation speed would not sediment mycobacteria very well and some bacteria would be lost during decanting the supernatant, which will affect the positivity rate.
- Regularly maintain and inspect - Unbalanced loads will cause vibration and possible tube breakage.

**Equipment - Centrifuges:**

- Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria.
  - Temperature increase during centrifugation increases the killing effect on mycobacteria which will decrease the positivity rate and increase time-to-detection.
  - If a refrigerated centrifuge is not available, avoid temperature build-up, especially if the room temperature is high. Add refrigerated (chilled) phosphate buffer before centrifugation which should help in keeping the temperature low. Other reagents used during the digestion/decontaminating step should not be refrigerated but kept at room temperature. Lower temperatures reduce the digestion decontamination process of NaOH-NALC.
- After centrifugation, allow tubes to sit for 5 minutes to allow aerosols to settle.

**Equipment - Autoclaves:**

- Sterilization will only result when conditions of time, temperature, pressure and humidity have been met.
- Mechanical recorder/indicators record the time-temperature profile attained during a cycle.
- Visual indicators chemically measure one or more physical conditions of the autoclave cycle.
- Spore testing (Biological indicators) are the only approved way to ensure sterility.
- Do not mix loads which require different exposure time and exhaust.
- Follow correct instructions for load preparation and placement in autoclave.
- Locate in or near the TB lab, do no transport bio-hazardous materials through public areas.
- Train staff in use and safety.
Equipment - Incubators:

- Connect to UPS or backup generators
- Monitor temperatures and CO2 content, if applicable, on a daily basis, including weekends and holidays
- Post acceptable ranges and document any corrective actions for temperature variances

Equipment - BSC and Practices

- Locate in far corner from entrance, away from traffic
- Train staff on correct practices
- Do not use a Bunsen burner or other heat generating equipment in cabinet
- Do not crowd with supplies during use, do not use to store supplies
- Schedule yearly checks and perform daily maintenance before use
- Do not use cabinet when alarm is sounding
- Use appropriate decontamination procedures on cabinet
- Require use of PPE for all possible aerosol generating procedures
- Disinfectants: use proper concentrations prepared daily for stability

Equipment - Automated Liquid Culture Systems:

- Perform validations
- Correlate with solid culture media/smear results
- Perform Quality Control on each new shipment/lot of reagent and media.
General Equipment

- thermometers, timers, Bunsen burners, vortex, pH meter, water baths, slide warmers, microscopes, balances:
  - Monitor temperature of slide warmers and water baths
  - Keep copies of equipment maintenance records performed by outside department or company
  - Calibrate pipettes, thermometers and balances using NIST-certifiable (up-to-date) references
  - Calibrate timers (not watches/wall clocks) against NIST or certifiable reference

Sputum Digestion/Decontamination

- Three important aspects must be considered:
- Specimens must be homogenized to free the bacilli from the mucus; the milder the better
- Neither homogenization nor decontamination should unnecessarily diminish the viability of tubercle bacilli
- Success of homogenization and decontamination depends on:
  - The greater resistance of tubercle bacilli to strongly alkaline or acidic digesting solutions
  - The length of exposure time to these agents
  - The temperature build-up in the specimen during centrifugation
- The efficiency of the centrifuge used to sediment the tubercle bacilli

Sputum Digestion/Decontamination

- Process sputums as soon as possible after collection to minimize overgrowth of contaminants and/or normal bacteria. Refrigerate specimens during shipment and if testing is delayed
- Process entire sputum specimen, do not attempt to select portions. If greater than 10 ml, use more than one tube to process
- Do not pool sputum specimens. Risk of cross-contamination
- Processing method must be compatible with the culture medium.
- Variances to accepted common practices must be validated.
- Use blood agar plate or other suitable media for checking contamination
  - Inoculate 4–6 decontaminated sputum specimens in addition to inoculating mycobacterial media. Numbers of contaminants that grow after 48 h of inoculation at 35°C should be minimal to none.
Sputum Digestion/Decontamination
Sodium hydroxide (Modified Petroff) Method:

Used widely in developing countries because of its relative simplicity and the fact that reagents are easy to obtain. NaOH is toxic, both for contaminants and for tubercle bacilli; therefore, strict adherence to the indicated timing is required. This may kill up to 60% of tubercle bacilli in clinical specimens. This initial kill is independent of additional contributory factors such as heat build up in the centrifuge and centrifugal efficiency. Sodium hydroxide should be used at the lowest concentration that will effectively digest and decontaminate the specimen. Use with a starting concentration of 3-4% NaOH. A higher concentration could be toxic to mycobacteria.

Sputum Digestion/Decontamination
N-acetyl-L-cysteine/2% NaOH Method:

When properly performed provides more positive cultures resulting in the killing of approximately 30% of tubercle bacilli. The digestant must be made fresh daily. When used with the rich MGIT broth and the nonselective nature of the MGIT indicator, strictly follow the recommended digestion-decontamination procedure to reduce the possibility of contamination. The initial concentration of NaOH is 4%. NaOH is bactericidal; NALC only liquefies the specimen and has no decontaminating properties. A high concentration of NALC or NaOH may result in false fluorescence in automated liquid culture systems.

Sputum Digestion/Decontamination
Digestion/Decontamination steps:

- **Timing**
  - Use a calibrated timer
  - Start timer for 15-20 minutes (up to 25 minutes maximum) after adding the NaOH-NALC solution to the first tube. Vortex lightly or hand mix/invert every 5-10 minutes or put the tubes on a shaker and shake lightly during the whole time.
- **Any variations from acceptable procedures must be validated**
- Quality control for process, media, reagents and equipment:
  - Include a negative control with each batch of specimens to be processed (5ml phosphate buffer)
  - Include a positive control (5 ml M. tuberculosis suspension at 0.5 McFarland diluted 1:500) with each new lot/shipment of media at a minimum. Position at the end of the batch.
  - Process negative and positive controls along with the clinical specimens, using the same digestion, decontamination and concentration methods.
  - A higher QC frequency is recommended for labs with fewer batches per week. These labs may be less proficient than labs with many batches per day.
Sputum Digestion/Decontamination

Digestion/Decontamination steps:

• Neutralization
  – At the end of the 15-20 minutes, add phosphate buffer (pH 6.8). Mix well (lightly vortex or invert several times). Addition of sterile water may not be a suitable alternative for the phosphate buffer.
  – The final pH of the specimen concentrate greatly affects the recovery and time-to-detection of mycobacteria. If water is used test sediment pH to ensure specimen is neutralized. High pH will lower the positivity rate and increase the time-to-detection of positive culture. High pH may cause transient false fluorescence. Keep the pH as close to neutral as possible. This step needs to be controlled very carefully.
• Carefully decant the supernatant into a suitable containing filled with mycobactericidal disinfectant. Use a method to reduce splashing- avoid overfilling waste containers.

Sputum Digestion/Decontamination

Digestion/Decontamination steps:

• Use disinfectant soaked absorbent material under the work area to immediately decontaminate any spills or droplets generated during the procedure.
• Pipettes (graduated serological versus disposable) – use appropriate size, the more often a container is entered the better chance of contamination.
• Mixing versus vortexing steps
  – Homogenization should occur by centrifugal swirling, and this swirling should not be vigorous enough to allow material to rise to the cap.
  – Invert the tube several times to mix the contents after the addition of sterile distilled water or buffer to reduce the continued action of the NaOH and lower the viscosity of the mixture.
  – Invert the tube so the whole tube is exposed to the NaOH-NALC mixture.
  – With NaOH-NALC digestion, do not agitate the tube vigorously. Extensive swirling causes oxidation of NALC and makes it ineffective.
• The exposure time to digestants/decontaminants has to be strictly controlled. Number of specimens processed per batch should not exceed capacity of centrifuge. Less than 12 specimens including controls, per batch is recommended.

AFB Smears:

• The acid-fast stain, either direct and/or concentrated should be used as an adjunct to culture. Definitive diagnosis required growth of the organism. A minimum of 5,000 – 10,000 bacilli per ml of sputum is required for detection by smear, whereas culture detects as few as 10 to 100 viable organisms.
• Include a positive and negative QC slide with each batch of stains.
  – Prepare smears from positive cultures on M. tuberculosis (H37Rv or H37Ra). Mycobacteria other than MTb Complex may also be used for positive control.
  – Bacterial suspensions such as E. coli may be used for a negative control.
• Training/Competency/Performance Monitoring of Staff
  – Minimum time of examination – 5 minutes per slide
  – Recheck Positive Smears by second technologist before reporting
AFB Smears:

- Smear prepared from direct or concentrated specimen
  - The overall sensitivity of the direct acid-fast smear has been reported to range from 22 – 80%.
  - Liquidation and concentration of sputum for acid-fast staining only may be conducted safely by first treating the specimens with an equal volume of 5% sodium hypochlorite solution (undiluted household bleach) and waiting 15 minutes before centrifugation. This treated specimen cannot be cultured.

- Fixation of slides
  - Allow the smear to air dry completely for 30 minutes. Heat fix by passing over a flame three to four times or by heating on a slide warmer at 65-75 C for 2-3 hours or overnight. Do not overheat or expose slide to UV light. Handle smears carefully since mycobacteria may still be viable.

- Use chlorine-free water (distilled or deionized) as chlorine may interfere in the fluorescence.

Culture/Media

- Use a liquid medium to isolate mycobacteria in conjunction with solid media. Liquid media is more prone to contamination with bacteria that are commonly present as normal flora. Growth on solid media is important for observation of colonial morphology and chromogenicity, for biochemical testing, and speciation, for the presence of more than one species of mycobacteria, or for a future reference.

- Egg based media supports the growth of most mycobacteria and permits niacin testing; contamination occurs more easily.

- Agar based has less contamination and earlier and easier visibility of colonial morphology but requires CO2 incubation; prevents overgrowth by contaminating bacteria or fungi.

- MGIT tubes which are instrument positive may contain one or more species of mycobacteria. Faster growing mycobacteria may be detected prior to slower-growing mycobacteria; therefore it is important to subculture positive MGIT tubes onto solid media to ensure proper identification of all mycobacteria present in the sample.

- Media – commercial and prepared require QC checks and documentation; lot numbers, exp, quality

- Documentation of the manufacturer’s QC procedures should be obtained by placing the lot number of each medium in a log book in addition to Certificates of Analysis.

- Every new lot of MGIT medium and every new lot of enrichment must be QC’d upon receipt and before routine use.

- Stains, reagents, antisera – labeled, storage, parallel testing, QC
Speciation

- Biochemical methods common.
- Levels reported for EQA must match those reported on patients.
  - Mycobacterium tuberculosis, Mtb complex
  - MOTT, use of growth characteristics (Rapid, photochromogen, etc)
- Quality Control – must be performed on each batch
- Incubation/Reading
  - If a specimen is suspected of containing mycobacteria which require an optimum temperature other than 37 C, (i.e. M. marinum, M. chelonae and M. ulcerans) then two sets of media should be inoculated, one at 37 C, the other in an separate incubator at 30 C. Specimens from skin and open wounds should always be inoculated using two sets of media.
  - Good light must be available to read solid agar cultures

Drug Susceptibility Testing

- Laboratories should be testing >50 TB isolates per year for proficiency. Less than that should be an indication that a referral laboratory should be found.
- QC using an ATCC (or equivalent) pan-susceptible organism must be performed on each run and with each new lot of reagents or medium. It is not necessary to include a resistant strain. Some MOTT can be used for resistance if needed.
- Only test pure cultures – MOTT is often resistant to antituberculin drugs

Reference Labs

- Plans often do not include a Reference laboratory for referral of organisms the laboratory is unable to identify.
- Designate an approved testing lab
- Use for validation of in-house procedures and when unable to identify isolates,
- Plans/arrangements made before needed to confirm MDR and XDR strains and provide additional drug susceptibility testing if needed.
Other Methods

• Some of this testing is as complex to perform as conventional culture and DST methods and requires skilled and well-trained laboratory personnel as well as adequate laboratory space and design with a dedicated PCR room. Problems with contamination are common especially with newly-trained staff.
• Molecular assays
• Rapid Diagnostics
• Quality Control – not clearly defined
• EQA – part of the identification algorithm, not specific testing methods.

Troubleshooting

These steps are often laboratory specific and many times technical in nature. They can often be resolved through observation and training efforts. Don’t rely solely on EQA which is infrequent and often lacks appropriate challenges.

Common indicators include:
• EQA Failures
• Change in Contamination Rates
• Low positivity rates compared to area
• Problems in smear to culture correlations

Laboratories Greatest Needs:

• Adequate supplies
• Administrative support
• Technical Training
• Safety Training
Resources Available:

- Networks
- SMILE
- www.pSMILE.org
- Other laboratories

References

Troubleshooting suggestions:  

Contamination:

Note: Liquid media is more prone to contamination than solid media.

Higher contamination may be due to:

• Use of non-sterile collection containers or materials such as pipettes, tubes, etc.
• Specimen transport/storage conditions after collection. Bacteria tend to overgrow in hot weather and are then hard to kill by routine decontamination methods without killing the mycobacteria
• Improper or under decontamination of specimen
• Very mucoid specimens that are hard to liquefy may result in high contamination

Troubleshooting suggestions:

Contamination (Cont.):

• If reagents are prepared, stored in bulk and used for long periods of time they may become contaminated. Leftovers should be discarded or resterilized.
• Particular days – situations such as construction pollution, dust storm, or dusty environment
• Particular season – high contamination rate in summer is common. Improve transport time and conditions
• One technician – some technicians are more experienced or careful than others
• Common contaminant – water or reagents
Troubleshooting suggestions:

Suggested steps for high contamination rates:

• Run a negative control to check for contamination during processing.

• Increase the NaOH concentration (not more than 1.5% final concentration in the specimen). Do not increase the exposure time to more than 25 minutes to NaOH-NALC solution.

• Increase the concentration of PANTA (BD BACTEC MGIT). Carefully evaluate this step as higher concentrations of some antimicrobials in PANTA may affect the growth of some species of mycobacteria other than tb.

• Do not change the NaOH concentration and the PANTA at the same time. Try one procedure at a time and document improvement of results.

Troubleshooting suggestions:

Suggested steps for high contamination rates (Cont.):

• If there seems to be a common source of contaminating bacteria, check sterility of all reagents.

• Try to reduce time between collection of the specimens and processing. If a specimen needs to be stored, use refrigeration.

• Transport specimens with ice and in an insulated chest, especially in hot weather climates.

• Invert the tube during the decontamination process to help decontaminate the inside surface of the top of the tube.
Troubleshooting suggestions:

Decrease or no recovery of mycobacteria:
Note: Specimens from chronically treated patients with drug-resistant TB take a longer time to grow
• Incubation
  – Temperature must be 37 ± 1 C for TB
  – Check temperature in different areas of the incubator
• Centrifugation
  – Loss of mycobacteria during centrifugation is significant. Generation of excessive heat will accelerate killing of the mycobacteria during centrifugation.
  – Speed must be 3,000 – 5,000 x g for recovery. Insufficient centrifugation speed may not bring down all the mycobacteria into the sediment.

Troubleshooting suggestions:
Decrease or no recovery of mycobacteria (Cont.):
• Decontamination procedure
  – Approximately 60 – 70% of the mycobacteria may be killed during processing.
  – Check purity and concentration of all reagents used
  – Use distilled water only for preparation of reagents
  – Reagents must be fresh
  – Reagents must be compatible with culture method/system
  – pH of the specimen, a high pH or very low pH may injure or kill mycobacteria during processing of the specimen or take longer for revival and growth of viable mycobacteria.
  – timing of exposure to decontamination reagent
  – If using MGIT, all negative tubes must be observed visually for turbidity and growth.
Troubleshooting suggestions:

Decrease or no recovery of mycobacteria (cont.):

• Delay in detection time using BD BACTEC MGIT
  
  – Digestion/Decontamination
    • Decrease the NaOH concentration and/or time of exposure.
    • High pH of the final inoculums will prolong the detection time
  
  – Incubator temperature - lower temperature will delay detection
  
  – PANTA—too high a concentration
  
  – Procedures check
    • Water should be distilled/deionized
    • All reagents should be sterile
    • All pipettes/tubes should be sterile
    • All inoculations should be in the Bio-Safety Cabinet
    • PANTA should be added to MGIT tubes just prior to inoculation
    • Open all tubes in the Bio-Safety Cabinet

Troubleshooting suggestions:

Decrease or no recovery of mycobacteria (cont.):

• Specimen Quality and Quantity
  
  – Too watery is not satisfactory. It is mostly saliva and may yield poor AFB recovery but would not contribute to contamination
  
  – If it is too mucoid it may need additional mucolytic reagent. If not completely liquified during the processing it may contribute to higher contamination. During the digestion procedure, if the specimen is not found completely liquified, add a small quantity of NAC powder.
  
  – Volume should be 2 – 10 ml. Lower volumes may yield fewer positive results. Higher volumes may contribute to higher contamination.
  
  – Must not be pooled which may result in high contamination.
Troubleshooting suggestions:
Decrease or no recovery of mycobacteria (Cont.):

• Specimen processing
  – Less than 15 minutes exposure is not long enough and will result in high contamination
  – More than 25 minutes would kill more contaminating bacteria but may also injure mycobacteria increasing time to detection
  – Mixing of specimen after adding digestion/decontamination reagent should be done two to four times
  – Too much vortexing oxidizes the reagent and makes it less efficient
  – Invert the tube a couple of times so the lip of the tube is well decontaminated
  – Insufficient mixing does not allow the reagent to mix with the specimen and may cause higher contamination
  – Addition of buffer is better than water. Water is not recommended for MGIT.
  – Keep the specimen tubes tightly closed and clean the outside before vortexing or shaking.
  – After vortexing, wait before opening the cap so that aerosol generated during the mixing settles down.

Troubleshooting suggestions:
Decrease or no recovery of mycobacteria (Cont.):

• Reagents
  – Check the concentration of in-house prepared reagents carefully
  – If sterilized in the laboratory, check autoclaving procedures and sterility of reagents
  – NaOH and Na-citrate reagents may be prepared, sterilized and kept for a long time but once NALC is added the solution is only fresh for 24 hours
  – Check NALC concentration to be sure it is at least 5%
  – An insufficient amount of NALC will not digest the specimens enough to allow the NaOH to come in contact with the contaminating bacteria
  – Presence of blood in a specimen would inactivate NALC
Troubleshooting suggestions:
Decrease or no recovery of mycobacteria (Cont.):
• Reagents (Cont).
  – Vigorous shaking may also inactivate NALC
  – Make sure NALC powder is not expired
  – Bulk reagents if used repeatedly become contaminated, cross-contamination may also occur
  – Reagents should be kept in aliquots, once used, the leftover discarded or re-sterilized if permitted
  – Avoid touching the specimen tube when adding the reagent or creating aerosols

Troubleshooting suggestions:
Decrease or no recovery of mycobacteria (Cont.):
• PANTA
  – Check storage conditions and expiry date of lyophilized PANTA
  – Check storage and timely use of reconstituted PANTA reagent
  – Check reconstitution of PANTA with sterile transfer device at correct volume
  – If PANTA is not completely dissolved, this will cause contamination problem
  – Growth supplement must be added inside the BSC to avoid introducing environmental contamination
  – PANTA should be freshly reconstituted and added to the MGIT tube on the day the medium will be inoculated
  – Be sure to mix MGIT tubes after all additions by inverting the tube.
Troubleshooting suggestions:

Decrease or no recovery of mycobacteria (Cont.):

• Specimen inoculation
  – Stagger tubes during processing, do not keep specimen tubes close to each other to avoid cross-contamination
  – Never inoculate media outside a safety cabinet
  – Open only one tube at a time