PCR Identification of Mycobacteria

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Iowa Overview

- 2016 Numbers
  - 3153 AFB cultures
  - 14% positivity rate
  - 1104 AccuProbes
    - 446 MTBC probes
    - 339 MAC probes
    - 289 MGO probes
Iowa TB Overview

- Positive TB cultures sent to California since 2015 for DST
- California forwards these isolates to Michigan for genotyping
- 50 isolates is the cutoff if laboratories perform AST testing in house
State Hygienic Laboratory Workflow

- Bactec MGIT 960
- AccuProbe Hybridization Assay for positive afb cultures (liquid/solid media) – MTBC, MAC, MGO
- NTM identification = mixture of 16s DNA sequencing and MALDI-TOF
Goals

• Replace AccuProbe hybridization assay with a more streamlined, rapid assay that can be linked directly into SHL’s LIMS system
• Report MTBC positive or negative culture result within 24 hours of MG
• Increase sensitivity of assay and range of organisms detected
• Expand number of technologists available to perform test
AccuProbe

- Nucleic acid hybridization test
- Uses a single-stranded DNA probe with a chemiluminescent label that is complimentary to the ribosomal RNA of the target organism
- Ribosomal RNA is released for the organism and the labeled DNA probe combines with the target organisms ribosomal RNA to form the DNA:RNA hybrid
- A separate selection reagent allows differentiation of the non-hybridized and hybridized probe
- The labeled DNA:RNA hybrid is measured in a Hologic luminometer
AccuProbe Process

- Build worksheet in LIMS
- Split liquid samples into micro centrifuge tubes and spin on benchtop centrifuge to concentrate
- Label the lysis tubes (one for each probe MTBC, MAC, MGO per sample)
- Add Reagent 2 and/or Reagent 1 to lysis tube
AccuProbe Process

- Add sample to each lysis tube
- Sonicate for 14 minutes followed by heat kill for 10 minutes
- Label detection tubes and transfer samples to each tube (again one tube for each probe)
- Heat tubes for 15 minutes
AccuProbe Process

- Organize tubes
- Read using Leader instrument (making sure to keep tubes in numerical order)
- Write numbers on receipt
- Write numbers on LIMS worksheet
- Transfer numbers into LIMS system
What’s the problem?

• AccuProbe requires six controls for each run
• Process is tedious and can/has led to cross-contamination and sample swap
• Sensitivity issues
• Reporting is a nightmare
• Test is expensive (labor) and reimbursement is low
The Solution

- MALDI has been hit or miss for SHL
- Real-time PCR
  - Fast
  - Cheap
  - Demonstrated use

**Evaluation of a Single-Tube Multiplex Real-Time PCR for Differentiation of Members of the Mycobacterium tuberculosis Complex in Clinical Specimens**

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Members of the Mycobacterium tuberculosis complex (MTBC) differ in virulence attributes, drug resistance patterns, and host preferences. The rapid differentiation of these species to determine nosocomial or human sources of tuberculosis disease or to direct treatment can benefit both public health and patient management. Commercially available assays cannot differentiate these species, and published assays have not been evaluated directly on clinical specimens. A real-time PCR assay for the differentiation of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canetti*, and *M. caprae* was developed. The presence or absence of regions of difference (RD) between the genomes of members of the MTBC allowed for the design of a single tube multiplex real-time PCR assay to differentiate these species. This assay measures the presence of RD1, RD2, RD4, RD9, RD12, and a region exterior to RD9 which is present in all MTBC members. To evaluate the performance of this assay, 192 clinical specimens positive for MTBC by real-time PCR were tested, resulting in a 94% correlation of the real-time PCR with the identification results obtained with cultured material. Additionally, 227 culture-negative positive cultures were tested, resulting in a 95% correlation between the methods. This real-time PCR is an inexpensive and rapid (2.5 h) method performed in a closed-format system and requiring minimal hands-on time that can be implemented in a clinical laboratory and used directly on clinical specimens.
RT-PCR Assay

• SHL currently validating Wadsworth real-time assay for detection of MTBC and MAC from positive MGITs and slants

• Will validate for direct detection of MAC and MTBC from clinical samples upon completion of the culture validation

• *Mycobacterium gordonae* is also under evaluation
PCR Validation

• 110 Mycobacteria species + 50 patient samples in real time
  – 20 MTBC isolates
  – 20 MAC isolates
  – 20 MGO isolates
  – 50 NTM and Actinomycetes
PCR Kill Study

• Kill Study Fail
  – Tested at 20 min, 30 min and 60 min at 80 degrees
  – Heavily inoculated cultured samples
  – Tested at lower temp to evaluate use for direct specimen and from positive culture

• Currently use 30 minutes at 100 degrees C
• Will need to lower this for direct specimen detection
PCR Extraction

• QIAmp DNA Mini Blood or Body Fluid kit
  – Bead based extraction
• Evaluated QIAcube
PCR Chemistry

• Trying to optimize primers and probes for culture based identification versus direct sample detection
• SYBER based
• Tested in duplicate from 100 nanomolar to 1000 nanomolar
• Culture is “hot” will require a minimum 1:10 dilution step
• Probably looking at 10 nanomolar of template
Mycobacterium gordonae

• Analyzing the 16s ITS region (between 16s and 23s)
• Issues with M. kansasii
• Doctor’s in Iowa have come to depend on this result as a rule-out for more serious infections caused by NTM infections
Updated Algorithm

- Reflex positive culture to MTBC and MAC real-time PCR (hopefully MGO)
- Samples negative by PCR will be analyzed using MALDI-TOF
- Further identification and characterization by 16s sequencing and NGS
Benefits of PCR

• MAC PCR detects more complex species than the probe assay
  – *M. avium*
  – *M. avium* subsp. *avium*
  – *M. avium* subsp *paratuberculosis*
  – *M. intracellulare*
  – *M. chimaera*
  – *M. arosiense*
  – *M. colombiense*
  – *M. marseillense*
  – *M. bouchedurhonense*
  – *M. timonense*
Benefits of PCR

• Cost
  – Accuprobe $75 per test (w/labor)
  – RT-PCR $25 - $40 per (w/labor)

• Turnaround time
  – SHL plans to run this assay Monday – Saturday as needed

• Staffing
  – SHL’s Molecular group can run assay during Microbiology busy season and vice versa
Benefits of PCR

• Sensitivity and Specificity
  – Concentration step for probes is replaced with a simple dilution
  – Increased sensitivity and specificity
• LIMS Connectivity
• Reimbursement
  – SHL currently receives $25 for a $75 AccuProbe test
• Replace Niacin/Nitrate for M.bovis/bovis BCG rule-out
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References


Questions