Victory from the jaws of defeat: molecular diagnosis of mycobacterioses from formalin-fixed, paraffin-embedded tissues

Julu Bhatnagar, Ph.D.
Christopher D. Paddock, M.D., M.P.H.T.M.

The findings and conclusions are those of the presenters and do not necessarily reflect the views of U.S. Department of Health and Human Services or the Centers for Disease Control and Prevention.

Infectious Diseases Pathology Branch, CDC

Provide tissue-based diagnosis of infectious diseases to:
- investigators across all centers at CDC
- public health staff at state and local health departments
- clinicians, community pathologists, medical examiners
- investigators in other countries
Diagnostic scenarios

- No cultures obtained during illness, or patient on antibiotic therapy prior to death
- No available specimens other than formalin-fixed tissues
- Specific assay only available at IDPB

Diagnostic resources

- Histopathology
- Clinical and epidemiologic information
  - syndrome
  - antibiotic history
  - demographics, season, geography, travel history, exposures
  - isolated vs. clustered disease
- Routine laboratory tests
- Other CDC laboratories
Challenges to PCR of formalin-fixed, paraffin-embedded tissues

- Fragmentation of nucleic acids limits size of target
- Formalin induces cross-linking between nucleic acids, addition of monomethyl groups, formation of methylene bridges
- Wild cards: type of fixative, duration of fixation, other tissue processing reagents (e.g., decal solutions), age of paraffin block

Benefits to PCR of formalin-fixed, paraffin-embedded tissues

- Biosafety, shipping, and storage concerns minimized
- A single specimen can be evaluated for multiple agents by multiple assays
- Retrospective analysis possible weeks-years after illness or death

*Mycobacterium* PCR requests: 2008-2013

![Chart showing Mycobacterium PCR requests from 2008 to 2013]
Oregon 2012: the case of the coughing bartender

- 28 y/o man found dead at home after 1 year of cough and marked weight loss
- ME suspected TB and obtained tissue for histology but not culture
- Contact tracing reveals multiple QFT-positive individuals

- DNA extracted from formalin-fixed, paraffin-embedded tissue
- PCR and sequencing confirm Mtb complex
  *Mycobacterium in IDPB*
- DNA provided to Mycobacteriology Laboratory Branch, CDC
- No mutations detected in any of 8 loci examined for antimicrobial resistance, including *rpoB* and *inhA*
Georgia 2012: TB or not TB?

- 34 y/o, HIV+, transgender man admitted to Grady Memorial Hospital for fever and altered mental status
- Found dead in hospital room from apparent suicide within hours of admission
- Medical examiner identifies para-aortic adenopathy at autopsy

- DNA extracted from formalin-fixed, paraffin-embedded tissue
- Negative PCR for Mtb complex
- Positive *Mycobacterium*-genus PCR assay; sequencing reveals *Mycobacterium avium* complex species
Missouri 2012: The curse of the Daintree Rain Forest

- 63 y/o man with large, non-healing ulcers on right calf and left foot
- Lesions began 2 months after emigrating to US from Queensland, Australia, where he had lived for 17 years
- No definite history of trauma; no response to multiple antibiotics; steroids initiated with progression of ulcers

- Impression smear of lesion reveals abundant acid-fast bacilli
- Cultures negative for mycobacteria
- Formalin-fixed, paraffin-embedded 4 mm punch biopsy specimen sent CDC
• DNA extracted from formalin-fixed, paraffin-embedded tissue

• Positive PCR for *Mycobacterium* genus; sequencing reveals highest similarity with *M. marinum* and *M. ulcerans*

• Positive *M. ulcerans* PCR assay targeting IS 2404; sequencing reveals 100% similarity with *M. ulcerans*
Ohio 2013: The Case of the Tiny Biopsy Specimen

- 18 y/o woman with nodular sclerosing Hodgkin’s disease
- Multiple splenic lesions identified by imaging; needle core biopsy reveals “a few acid-fast bacilli”
- Cultures for mycobacteria negative, PPD-negative, QuantiFERON negative
- Placed on clarithromycin/rifabutin/ethambutol for presumed MAC infection

- Negative acid-fast stain
- Negative IHC

- DNA extracted from formalin-fixed, paraffin-embedded tissue
- Negative PCR for Mtb complex Mycobacterium species
- Positive PCR for Mycobacterium genus; sequencing reveals highest similarity with *M. asiaticum*

Florida, 2010: The Case of the Unknown Infection

- 49 y/o woman with h/o lobular carcinoma in-situ develops 3-cm axillary adenopathy

- Outside pathology laboratory identifies necrotizing granulomas and "occasional small rod-like organisms in chains" with acid-fast stain; no growth of mycobacteria from aspirate

- Case sent for consultation; no acid-fast bacteria identified.....however, PCR of formalin-fixed specimen positive for Brucella

PCR assays available for bacterial pathogens

- Broad-range 16S rDNA
- Streptococcus pneumoniae
- Neisseria meningitidis
- Haemophilus influenzae
- Staphylococci (including MRSA)
- Streptococci pyogenes
- Mycobacteria group and M. tuberculosis complex
- Mycoplasma pneumoniae
- Legionella pneumophila
- Clostridia group and C. sordelli
- C. perfringens, C. difficile
- Coxiella burnetii
- Bartonella henselae
- Anaplasma phagocytophilum
- Tropheryma whipplei
- Capnocytophaga
- Chlamydia/chlamydophila
- Enzcheta chaffeensis
- Escherichia coli
- Fusobacterium
- Helicobacter
- Klebsiella
- Legionella
- Listeria
- Moraxella catarrhalis
- Pseudomonas
- Rhodococcus equi
- Rickettsia species
- Streptobacillus moniliformis
- Yersinia enterocolitica
- DNA extracted from formalin-fixed, paraffin-embedded tissue
- Negative PCR for *Mycobacterium* genus
- Negative IHC for *Brucella* species, but positive IHC for *Bartonella henselae*
- Positive PCR for *B. henselae*

### Identification of *Mycobacterium* spp. from Formalin-Fixed, Paraffin-Embedded Tissues by PCR and Sequencing

**Outline**

- Development and validation of molecular diagnostic assays for the identification of *Mycobacterium* spp. from formalin-fixed, paraffin-embedded (FFPE) tissues
- Results of the testing performed on FFPE tissues using these assays
- Comparison of various tissue-based diagnostic assays (AFB, IHC and PCR) for this series of cases
- Correlation of molecular results with clinical history and histopathology

### Rationale

Molecular identification of *Mycobacterium* spp. from tissues is useful-

- No other appropriate specimen is available for the conventional diagnostic methods (culture, biochemical analysis)
- Clinical suspicion of slow growing or difficult to culture *Mycobacterium* spp. and rapid diagnosis is required
- Improve sensitivity and specificity of tissue-based detection
- Differentiation of MTB vs. NTM
- Identification of *Mycobacterium* species (>160 species)
- No commercially available PCR assay that is validated for tissue specimens
Jaws of defeat…. to ….victory stories

Development of Molecular Diagnostic Assays

- Problems inherent with FFPE tissues
  - Degradation and fragmentation of DNA (<600-bp)
    - Delay before fixation (if not quickly moved to -20ºC)
    - Prolonged fixation in formalin (>24-48 hours)
    - Fixative not appropriate for molecular analysis (formaldehyde)
    - Size of tissue (too small or too big)
  - Fixation-induced cross-linking between nucleic acids and proteins (difficult tissues - rich in proteins)
  - Low DNA concentration (tiny tissue samples, fibrous tissue)
  - Mycobacterium has a robust, thick and waxy cell wall that is rich in lipids/mycolic acids - hinders cell lysis

DNA Extraction from FFPE Tissues

- DNA Extraction
  - QIAamp DNA Mini kit and QIAamp DNA Micro kit (biopsies)
  - QIAGEN-column based kits (different binding capacities)
    - Paraffin section (16 µm)
    - Xylene (1X)
    - Deparaffinise (5 min)
    - Ethanol wash (2X)
    - Air dry
    - Addition of ATL buffer and proteinase K
    - Homogenize with disposable pastels
    - Tissue digestion with proteinase K
    - Incubate overnight at 56 ºC
    - Addition of AL buffer
    - Incubate at 70 ºC/10 min
    - Tissue extraction protocol
    - Elute DNA in 50 µl of AE buffer

DNA Quality Control

- To evaluate the quality of DNA, a duplex house-keeping gene PCR assay is performed on each sample
- The assay targets a 200-bp segment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and a 500-bp segment of b-globin gene
- Helpful for the assessment of the level of fragmentation and the presence of PCR inhibitors - to avoid false negative PCR results
- To avoid false positive PCR results - separation of pre- and post PCR work areas, unidirectional workflow

Mycobacterium spp. PCR Assays

- Multiple, stepwise, Mycobacterium genus and species specific conventional PCR assays using High Fidelity PCR kit (Roche)
**Positive and Negative Controls**

**List of Positive Controls (FFPE cell culture controls)**
- M. tuberculosis (ATCC 25177)
- M. avium (ATCC 25291)
- M. fortuitum (ATCC 6841)
- M. gordonae (ATCC 14470)
- M. chelonae (ATCC 35752)
- M. abscessus (ATCC 19977)
- M. kansasi (ATCC 12478)
- M. scrofulaceum (ATCC 9981)
- M. mucogenicum (ATCC 49650)
- M. immunogenenum (ATCC 700500)
- M. marinum (ATCC 927)
- M. triplex (ATCC 700071)
- M. interjectum (ATCC 51457)
- M. genavense (ATCC 51233/51234)
- M. parascrofulaceum (CIP 108112)
- M. leprae (culture confirmed case)

**List of Negative Controls**

(FFPE cell culture controls and confirmed cases)
- Nocardia spp.
- Bartonella henselae
- Histoplasma capsulatum
- Blastomyces dermatitidis
- Aspergillus spp.
- Listeria monocytogenes
- Legionella spp.
- Rhodococcus equi
- Coccidioides
- Caviella bumeri
- Pseudomonas aeruginosa
- Burkholderia spp.
- S. aureus
- S. pneumoniae
- S. pyogenes
- Clostridium perfringens
- Francisella tularensis
- Yersinia pestis
**Sensitivity and Specificity of PCR Assays**

- **Specificity**
  - *Mycobacterium* genus specific PCR (16S rRNA gene)- 100%
  - *M. tuberculosis* complex PCR assays (IS6110 and hsp65)- 100%
  - *Mycobacterium* genus specific PCR (rpoB gene)- 89%

- **Sensitivity**
  - *Mycobacterium* genus specific PCR (16S rRNA gene)- 97%
  - *M. tuberculosis* complex PCR assays IS6110-92%; hsp65- 96%
  - *Mycobacterium* genus specific PCR (rpoB gene)- 100%

**Sequencing of PCR Products**

- All amplified PCR products - analyzed on 2% agarose gel and extracted from the gel (QIAGEN kit)
- Directly sequenced on a GenomeLab Genetic Analysis System (Beckman Coulter)
- Sequence analysis performed - using CLC software, search for homologies with the known sequences - using the NCBI BLAST

**Testing Performed on FFPE Tissue Specimens of Case–patients using Mycobacteria PCR Assays**

- **Patients Specimens**
  - DNA was extracted from FFPE tissue specimens of 124 cases
  - Case definition: Clinical and histopathological findings suggestive of Mycobacterial infection
  - The cases were submitted to the Infectious Diseases Pathology Branch, CDC from 2003-2013 for diagnostic consultation
  - The majority (75%) of the cases were more recent (2009-2013) cases
  - Based on the availability of tissue specimens, the older cases were retrospectively tested by newly developed methods
  - Ninety-four percent of cases were from USA

**Results**

- Out of 124 cases, *Mycobacterium* spp. were detected in 60 (48%) cases
- Other pathogens were detected in 9 (7%) cases – *Nocardia, Bartonella, Aspergillus, Pneumocystis, Legionella, S. mitis, Burkholderia*

**Table 1. Demographics and laboratory findings of 60 positive cases**

<table>
<thead>
<tr>
<th>Demographics and Laboratory findings</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>38 (63%)</td>
</tr>
<tr>
<td>Median Age</td>
<td>45 (age range, 3 months-62 years)</td>
</tr>
<tr>
<td>Pediatric (&lt;18) cases</td>
<td>7 (12%)</td>
</tr>
<tr>
<td>Fatal</td>
<td>14 (23%)</td>
</tr>
<tr>
<td>Laboratory findings (IDPB)</td>
<td></td>
</tr>
<tr>
<td>Mycobacteria PCR positive</td>
<td>60 (100%)</td>
</tr>
<tr>
<td>Mycobacteria IHC positive</td>
<td>37 (61%)</td>
</tr>
<tr>
<td>AFB positive</td>
<td>32 (53%)</td>
</tr>
<tr>
<td>Outside CDC lab. findings</td>
<td></td>
</tr>
<tr>
<td>AFB positive (based on available records)</td>
<td>31 (51%)</td>
</tr>
<tr>
<td>Culture positive (based on available records)</td>
<td>7 (12%)</td>
</tr>
<tr>
<td>Species identified</td>
<td></td>
</tr>
<tr>
<td>*M. tuberculosis complex (MTC) species</td>
<td>24 (40%)</td>
</tr>
<tr>
<td><em>Non tuberculosis Mycobacterium spp.</em></td>
<td>36 (60%)</td>
</tr>
</tbody>
</table>
Results

Table 2. List of Non-tuberculous Mycobacterium (NTM) species identified

<table>
<thead>
<tr>
<th>Non-tuberculous Mycobacterium (NTM) Species Identified</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. avium complex (MAC) sp.</td>
<td>18 yrs of age</td>
</tr>
<tr>
<td>M. marinum</td>
<td>4</td>
</tr>
<tr>
<td>M. leprae</td>
<td>2</td>
</tr>
<tr>
<td>M. interjectum</td>
<td>2</td>
</tr>
<tr>
<td>M. haemophilum</td>
<td>2</td>
</tr>
<tr>
<td>M. abscessus</td>
<td>1</td>
</tr>
<tr>
<td>M. genavense</td>
<td>1</td>
</tr>
<tr>
<td>M. asiaticum</td>
<td>1</td>
</tr>
<tr>
<td>M. parascrofulaceum</td>
<td>1</td>
</tr>
<tr>
<td>M. ulcanean</td>
<td>1</td>
</tr>
<tr>
<td>NTM</td>
<td>1</td>
</tr>
</tbody>
</table>

Clinical correlation - NTM species

<table>
<thead>
<tr>
<th>Mycobacterium app. identified</th>
<th>Epidemiologic Features</th>
<th>Clinical &amp; Histopathologic Features</th>
<th>Published Reports</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. marinum (n=4)</td>
<td>Exposure to fish farm, deep sea fishing (n=2), gardener (n=1), Carpel tunnel (n=1)</td>
<td>Granulomatous acute &amp; chronic pyoderma (n=2), subcutaneous nodules (n=1), granulomatous granuloma (n=1)</td>
<td>Chopra N et al, J Clin Rheumatol. 2002 Oct;8:265-8. (5 case reports)</td>
</tr>
<tr>
<td>M. parascrofulaceum (n=1)</td>
<td>Correct injection of unknown origin (adcomm 87?) near nodular area</td>
<td>At 56 yrs, nodules on glabella, non-caseating granulomas</td>
<td>Zong W, et al, Ther Clin Risk Manag. 2012 (skin)</td>
</tr>
</tbody>
</table>
Summary

- DNA extraction methods were optimized for FFPE, archived, autopsy and biopsy tissue specimens (some stored for 10 years)
- Out of 124 cases, 60 (48%) were positive by *Mycobacterium* genus PCR assays, 37 (30%) were positive by IHC and 32 (26%) were AFB positive
- For this series, *Mycobacterium* genus PCR assays were the most sensitive assays and identified *Mycobacterium* species in 58 cases.
- In 23 cases (38% of positive cases), *Mycobacterium* spp. were detected only by PCR
- Causative organisms of the disease were detected in 55% of cases (*Mycobacterium* sp. in 48% & other pathogens in 7% cases)

Summary

- *M. tuberculosis* complex species were identified in 40% of positive cases, while NTMs were identified in 60% of positive cases by PCR and sequencing
- Major proportion (30%) of identified NTMs was *M. avium* complex species
- Other uncommon/rare species (*M. asiaticum, M. ulcerans, M. wolinskyi, M. parascrofulaceum*) were also identified and clinico-pathologic correlation was established for the majority of the cases
- In addition, *M. lepra* was also identified in two case by *M. leprae* specific PCR and sequencing

Conclusions

- PCR and sequencing using the FFPE tissues helps in rapid identification of *Mycobacterium* species which, in turn, can aid in timely selection of specific antimicrobial therapy
- Tissue diagnosis, using the combination of PCR, IHC and AFB stains, also helps to correlate association of NTM species with specific clinical manifestations and to better characterize the pathogenic potential of uncommon species
- The PCR assays can also be used for the retrospective analysis of older cases and sequence information obtained can be useful for the epidemiologic investigations
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