Necropsy of GEM: The good, the bad, and the ugly

Kelli Boyd DVM, PhD, DACVP
Department of Pathology
Vanderbilt University Medical Center

July 21, 2009
Overview

- Necropsy Evaluation
- Tissue collection
- Fixation
  - Considerations for ancillary testing
- “Normal Pathology”
  - Stain Background Pathology
  - Unique mouse lesions
- Resources
There is always something new or unexpected

There is always another way
Necropsy Evaluation

- General principles
- Observation of Live animal
- Terminal blood collection
- External examination
- Evaluation of internal organs
- Lesion description
Autolysis happens!

- If the animal is found dead in the cage in the morning it will most likely be too autolyzed for necropsy.

- Refrigeration is good
  - It slows down autolysis.

- How long is too long between death and necropsy
  - If not refrigerated >4 hours is too long.
  - If refrigerated about 6 hours max for most tissues.

- The Gastrointestinal tract autolizes quickly b/c of bacterial load;
  - If doing GIT work, fixation should immediately follow death.

- If you sac the animal and you want to perform histology on the tissue, put the tissue in fixative immediately.

- **Never** put the carcass in the freezer or put tissues in the freezer prior to histology.
  - Note: This does not include flash freezing for frozen sectioning.
  - Use 10x the amount of fixative as tissue.
Live animal observation
Terminal blood collection

Retro-orbital

Intracardiac
External examination

- Skin
- Oral cavity
- Eyes
- External ear
- Reproductive tract
- General body condition
Internal organ evaluation

- Performed the same way every time
- Record findings on each individual animal
- Knowledge of normal mouse anatomy is essential
- Knowledge of normal diseases in background strains is essential
- Accurate tissue identification and lesion description
- Organ weights
Opening the mouse

Pin mouse
Wet fur with alcohol

Use forceps to raise skin
Using scissors cut opening in skin
Create pocket in the subcutaneous tissue along each rear leg
Cut skin and peel back

Create pocket in the subcutaneous tissue to the anterior of the mouse
Cut skin along the pocket and peel back
Salivary glands
Preputial glands
Mammary tissue (aka inguinal fat pad)
Prepuce
Scrotum
Inguinal lymph node
Inguinal lymph node
Preputial glands
Scrotum
Inguinal lymph node
Inguinal lymph node
Tissue Handling

• Collection
  – Tissue samples should not be greater than 1cm thick for adequate fixation
  – Try to hold the connective tissue around the tissue to prevent crush artifact
• Lung inflation
  – Infuse 1ml-1.5ml of fixative via the trachea
• Swiss roll
  – Flush intestine with fixative
  – Roll in cassette
Whole head sections

- Fix head overnight in 10% formalin
- Decalcify head for 24-48 hours in 23 % formic acid
  - TBD-2 solution by Thermo-Shandon
  - The histologic appearance of tissues left in TBD-2 up to 72 hours is okay. However, antigen integrity for immunohistochemistry or targets for in situ hybridization may be compromised.
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Heart, thymus, skeletal muscle</td>
</tr>
<tr>
<td>2</td>
<td>Tongue, trachea, thyroid, esophagus, lung, mediastinal LN</td>
</tr>
<tr>
<td>3</td>
<td>Kidneys with adrenals attached</td>
</tr>
<tr>
<td>4</td>
<td>Liver, spleen</td>
</tr>
<tr>
<td>5</td>
<td>Peripheral lymph nodes</td>
</tr>
<tr>
<td>6</td>
<td>Salivary glands, stomach, pancreas</td>
</tr>
<tr>
<td>7</td>
<td>Small Intestines (swiss roll)</td>
</tr>
<tr>
<td>8</td>
<td>Cecum, Colon, Rectum</td>
</tr>
<tr>
<td>9</td>
<td>Urogenital tract</td>
</tr>
<tr>
<td>10</td>
<td>Skin</td>
</tr>
<tr>
<td>11</td>
<td>Whole Head sections</td>
</tr>
<tr>
<td>12</td>
<td>Sternum (Bone marrow)</td>
</tr>
</tbody>
</table>
There’s no time for a necropsy. What can I do?!
Fixation

- Most Common Types used
  - 10% Neutral Buffered Formalin
  - 4% paraformaldehyde
  - Bouins fixative
  - Davidson’s solution
  - Fekete's acid-alcohol-formalin
  - Glutaraldehyde (most commonly 4%)

- Immersion fixation
- Perfusion
10% NBF

- Standard fixative
- Easy to acquire and store
- Works for most applications
- Recent manuscript reports with HIER
  - long term fixation up to 6 weeks can be overcome for 61 common antibodies
  - Tissues were stained same day of sectioning
  - Dog mainly small number of cat samples
  - CK7, HMWCK and Laminin diminished
    - Webster et al.
      - Journal of Histochemistry April 2009
4% paraformaldehyde

- Commonly used in research
- Must be prepared fresh
  - >48 hours old = DON’T USE
- Works for most applications
- May be prepared with a variety of buffers to fit application
- For routine fixation basically the same as formalin
Bouins

- Slightly acidic
- A little better penetration than formalin
- Often used for:
  - Embryo
  - Reproductive tract
- >48 hrs in Bouin’s Tissues will become brittle
  - Switch to 70% alcohol
- Order pre-made
  - Picric acid is explosive
  - EHS won’t be happy
- Can inhibit IHC
• Fetke’s Acid Alcohol
  – Used at Jax lab
  – Good for skin and eyes
  – Tissues must be transferred to 70% alcohol after 48 hours
• Davidson’s Solution
  – Primarily used for eyes
  – Softens the lens
    • Makes grossing and sectioning easier
• Glutaraldehyde
  – Fixative for Electron Microscopy
  – 4% works well
  – Can be fixed after formalin or Paraformaldehyde but not optimal
  – Glut fixed tissues not great for histology
• Immersion fixation
  – Fine for most applications

• Perfusion fixation
  – Preferred in many research applications
    • Central and peripheral nervous system
    • Some Electron Microscopy studies
  – Methods
    1. Anticoagulant (pre-treat animal or mix with buffer)
    2. Buffer to flush system
    3. Fixative of choice
General principles for perfusion

• Buffer used for initial flush
  – Routine histo
    • Normal saline or Phosphate buffered saline +/- heparin
  – Electron microscopy
    • 0.1M cacodylate buffer with 4% sucrose, 0.2 calcium chloride
    OR
    • 0.1M Phosphate buffered saline

• Depending on application may need to be ice cold

• Flush pre-wash buffer until the exiting fluid is clear
  – For mice this volume is typically between 30-50 mls
General principles for perfusions

• Fixative
  – Routine processes
    • 10% NBF works well
    • 4% paraformaldehyde
• Tissue needed for routine histology and EM
  – 4% para in 0.1m phosphate
    Or
  – 4% paraformaldehyde ;4% sucrose in 0.1M cocodylate buffer
  – Then fix over night in 4% glutaraldehyde
General principles for perfusions

- TO PUMP or NOT TO PUMP
  - Manual perfusion via syringe
  - Mechanical perfusion using pump
  - Gravity* Best method

- Artifacts
  - Pressure to high (usually with Manual and Mecanical )
    - Vessel dilation and rupture
    - Cell and organelle swelling and rupture can occur
  - These artifacts are especially problematic for EM studies
Cryosectioning

• Cryosectioning (section frozen tissues)
  – Embed tissue in OCT or other cryomatrix
  – Freeze on dry ice or liquid nitrogen
Placing tissues in cassettes

- Tissue should not be thicker than cassette
  - $>0.5$ cm
- Tissue should not fill the entire cassette
- Use a pencil to write on cassette
  - Sharpie or other markers will wash off during processing

Questions???
Find your histologist!
Lesions considered normal for the particular background strain
- C57BL/6- microphthalmia, hydrocephalus, dermatitis, osteoporosis, lymphoma or histiocytic sarcoma, amyloidosis, acidophilic crystalline/macrophage pneumonia, hyalinosis
- 129- teratomas, Harderian gland tumors, lung tumors, nephropathy, acidophilic crystalline/macrophage pneumonia, hyalinosis
- FVB- retinal degeneration, seizures, lung tumors, acidophilic macrophage pneumonia
- Retinal degeneration-FVB,C3H, CBA, SJL
- Absent corpus callosum- Balb-c
- Distrophic cardiac calcification- Balb-c, C3H, DBA
Respiratory infection with *Francisella novicida* induces rapid dystrophic cardiac calcinosis (DCC)

Kimberly M. Roth, Steve Ogihara, Anjali A. Sabatkar, John S. Gunns, Nico van Rooijen & Asbey R. Satozka

Department of Microbiology, The Ohio State University, Columbus, OH, USA; Department of Pathology, The Ohio State University, Columbus, OH, USA; Center for Microbial Interface Biology, The Ohio State University, Columbus, OH, USA; and Department of Cell Biology and Immunology, Free University, Amsterdam, The Netherlands

Correspondence: Asbey R. Satozka, Department of Microbiology, The Ohio State University, 100 West 12th Avenue, CB 1281, Columbus, OH 43210, USA; Tel.: +1 614 292 7240; Fax: +1 614 292 2840; E-mail: satozka.8@osu.edu

Received 1 August 2007; revised 9 January 2008; accepted 21 January 2008. First published online 9 April 2008.

**Abstract**

*Francisella tularensis* causes pulmonary tularemia and death in humans when left untreated. Here, using a novel aerosol infection model, we show that acute pulmonary *Francisella novicida* infection not only causes pneumonia and liver damage, but also induces dystrophic cardiac calcinosis (DCC) in BALB/c mice. C57BL/6 mice also develop pneumonia and hepatic damage, but fail to develop DCC. Development of DCC in BALB/c mice is associated with significant induction of RANKL but not osteopontin in their organs. Depletion of lung macrophages prior to infection markedly reduces periostitis and calcification in BALB/c mice but does not increase their susceptibility to infection.

**Introduction**

Although the natural incidence of pulmonary tularemia is extremely low, the high infectivity, rapid dissemination, and severity of the disease caused by *Francisella tularensis* dictates its classification as a Group A bioterrorism agent (Davis et al., 2001). Acute exposure of mice to *Francisella novicida*, a subspecies of *F. tularensis*, which is nonpathogenic for humans, results in fatal pneumonia and liver pathology that mimics respiratory tularemia in humans.

*Francisella tularensis* DNA has been detected previously in the cardiac tissues of infected patients and endothelitis has been observed in rhesus monkeys (Ball et al., 1973; Langer et al., 2004), but there are no reports of pericardial calcification following a natural infection with *Francisella*. Dystrophic cardiac calcinosis (DCC) results from injury to the heart in certain stressed strains of mice such as BALB/c and DBA/2, and is characterized by the formation of calcified plaques on the pericardium (O’Regan & Rahman, 2003). Several studies have linked calcification in mice with the *Ducd2* genetic loci as well as osteopontin and RANKL, two cytokines which play an integral role in both cellular immune responses and inflammation (O’Regan & Rahman, 2003; Alberachrou et al., 2004; Collins-Osobny, 2004). While some reports suggest that osteopontin is involved in inducing DCC (Alberachrou et al., 2004), others indicate that osteopontin is required for its protection (Giacchetti et al., 2005). RANKL is produced during inflammation and is a natural ligand for RANK, which is expressed on the surface of macrophages and dendritic cells. Unlike osteopontin, it is widely accepted that RANKL promotes calcification via its interaction with RANK. DCC has many causes, including hormonal treatments, and chronic viral and bacterial infections; however, it has never been reported in acute infection (O’Regan & Rahman, 2003).

In this study, we found that unlike C57BL/6 mice, BALB/c mice produce high levels of RANKL, but fail to up-regulate osteopontin production in their heart and lung tissue during acute pulmonary infection with *F. novicida* and develop DCC. We also show that pulmonary macrophages are involved in the pathogenesis of DCC during *F. novicida* infection.
Precancerous lesions upon sporadic activation of beta-catenin in mice.

Coste I, Freund JN, Spaderna S, Brabletz T, Renno T.
## Teratomas in the Skin

### Table 1. Neoplastic Growth Phenotypes of mPer2m/m Mice

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>mPer2m/m Mice (18 months old) (n = 20)</th>
<th>Wild-Type Mice (18 months old) (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivary gland hyperplasia</td>
<td>20 (50%)b</td>
<td>0</td>
</tr>
<tr>
<td>Teratoma in male mice</td>
<td>10 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Hair graying 6 months after IR</td>
<td>3 (15%)</td>
<td>0</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Angiosarcoma</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(C) Gross photo of teratoma in an unirradiated male mPer2m/m mouse.

(D) Mature cystic teratoma of hyperkeratotic skin with subaceous glands shown in (C) (10 × 10).

**Fu et al, CELL 111: 41, 2002**
Avoid interpretation pitfalls

• Set up appropriate controls
• Know what is normal for your mouse strain
  (C57BL/6, FVB, etc.)
• Find a Comparative Pathologist
• Utilize online resources
Online links-
Conclusion

• There are many techniques to accomplish similar goals
• Use a systemic approach to evaluate your mouse
• Remember mouse strains are unique and understanding the influence of background strain is important
• Find an expert if you need help
Acknowledgements

• SJCRH Vet Path Core
  – Brenda McGowan
  – Debra Williams
  – Hermitta McLaurine
  – Pam Johnson
  – Lourie West
  – Joe Emmons

• SJCRH EM Core Facility
  – Jackie Williams
  – Fara Sudlow
  – Sharon Frase

• Vanderbilt University IHC Core
  – Frances Shook
  – Melissa Downing
  – Division of Animal Care
    – Dr. Ken Salleng
    – Dr. Troy Apple
  – Photography
    – Michelle Endres
The end!
Questions?

Kelli.I.boyd@vanderbilt.edu
Useful Links

Mouse Pathology

- Mouse anatomy
  http://www.informatics.jax.org/cookbook/chapters/contents2.shtml
- Normal mouse histology
- Virtual mouse necropsy
  http://www.geocities.com/virtualbiology/
- Diseases of Laboratory animals
  http://www.radil.missouri.edu/info/dora/Dora.htm
- The Center for Genomic Pathology
  http://ctrgenpath.net/
- Revised guides for organ sampling and trimming in rats and mice published in 2003 and 2004 in three parts in Experimental and Toxicologic Pathology.
  http://www.item.fraunhofer.de/reni/trimming/index.php
- Proliferative lesions in the lung

Atlases

- Atlas of Laboratory Mouse Histology
  http://www.ctrgenpath.org/static/atlas/mousehistology/
- MBL – mouse brain library
  http://www.mbl.org/procedures/procedure.php
- Edinburg mouse atlas project
  http://genex.hgu.mrc.ac.uk/

Resources

- Mouse nomenclature
- Coat colors of mice
  http://www.informatics.jax.org/wksilvers/
- Mouse Genetics
  http://www.informatics.jax.org/silver/
- European tumor database
  http://www.pathbase.net/
• MMHCC Mouse Models of Human Cancer Consortium
  http://emice.nci.nih.gov/
• Mutant mouse regional resources supported by NCRR-NIH
  http://www.mmrrc.org/
• The trans NIH mouse initiative
  http://www.nih.gov/science/models/mouse/
• Riken mouse mutagenesis project
  http://www.brc.riken.jp/lab/gsc/mouse/
• North American Conditional Mouse Mutagenesis project
  http://norcomm.phenogenomics.ca/index.htm
• KOMP NIH knock out mouse project
• KOMP data coordination center
  http://www.knockoutmouse.org/
• The mouse as a model for human biology: a resource guide for complex trait analysis
  http://www.nature.com/nrg/journal/v8/n1/box/nrg2025_BX1.html
• Complex trait consortium
  http://www.complextrait.org/
• Understanding human disease through mouse genetics
  http://www.eumorphia.org/

Databases

• Mouse phenome database
  http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home
• MGI Mouse Genome Informatics
  http://www.informatics.jax.org/
• MTB Mouse Tumor Biology Database
  http://tumor.informatics.jax.org/mtbwi/index.do