Mycobacterium avium subsp. paratuberculosis - Studies on isolation and detection in milk, feces and cow tissues

Dr. Lucy Mutharia

Collaborators:
Dr. Joseph Odumeru & Dr. Anli Gao (Lab Services Division)
Ms. Melinda Raymond (MCB)
Dr. Colin Gill (Agri-food Canada Lacombe Research center)
Presentation Outline

• MAP bacterium
• MAP is a pathogen of many animal species
• Research objectives
  – Does MAP survive pasteurization? If so are live MAP present in retail milk?
  – What is the distribution of MAP bacteria in tissues of JD-positive animals?
MAP bacterium

- Short acid-fast staining bacilli
  *Thick waxy coat* with Mycolic acids

- Strictly a pathogen
  *Survives outside of host*

- 3 major types  
  (Semret et al 2005)
  
  S (Sheep) or **Type I**
  
  C (cow) or **Type II**
  
  I (intermediate) or **Type III**

- Belongs to the *M. avium* complex, but
  *Extremely slow growing*

  *Mycobactin*-depend for culture
  Subspecies-specific DNA sequences,
  IS900, *hspX*
I. MAP & Johne’s Disease

Faecal-Oral transmission

{Faeces, colostrum, milk, water, pasture}

Months to years

Advanced / clinical JD

Wasting, Diarrhoea, MAP in faeces & milk, loss of productivity

(Wiki)

(Doctor D. Butler OVC)
MAP & Human Crohn’s Disease

• Highly controversial topic

• Pros
  - Similarity in symptoms & gross pathology between JD and CD
  - Goats infected with a human MAP isolate developed JD
  - MAP was cultured from blood, breast milk and intestinal tissues of patients with CD
  - In some CD patients, antimycobacterial (MAC) antibiotics achieved partial or full remission.

Cons:
  - No increase in CD rates among rural populations exposed to JD-positive animals
  - MAP has been isolated from patients with diseases other than CD

Project 1: Are live MAP bacteria present in Ontario retail milk?

**Background:**

- MAP were more thermotolerant than other mycobacteria.  

- **3% to 5% of cow MAP** and **24.8 – 31.4 human MAP survived** exposure to HTST pasteurization  
  (Chiodini & Hermon-Taylor 1993)

- Several studies reported culture of MAP from commercially pasteurized retail milk
  
  - **1.8%** (of 567 samples) in the UK  
    (Grant et al 2001)
  
  - **2.8%** (of 702 samples) in the USA  
    (Ellingson et al 2005)

- At **$>10^4$ cfu/ml** MAP in milk, some MAP will escape killing by HTST (high temp short term) pasteurization (**72°C for 15-25sec**)  
  (Grant et al 1996)
Experimental approach

• Optimize recovery of MAP cells from milk
  – whole milk, cream and pellet fractions
• Optimize extraction of MAP DNA for use in PCR
  – IS900 and hspX
• Determine PCR assay sensitivity
  – Direct vs. nested PCR
• Screen raw and retail milk for MAP by PCR and culture
Results
MAP preferentially partition to the cream fraction

a) The best recovery was from the combined pellet + cream
Efficiency of preparation of MAP template assessed by IS900-based direct PCR

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Combined procedures used in preparation of template DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Bead Beating</td>
<td>✓</td>
</tr>
<tr>
<td>Freeze-thaw (5 cycles)</td>
<td></td>
</tr>
<tr>
<td>Lysis Buffer</td>
<td></td>
</tr>
<tr>
<td>Boiling (20 min)</td>
<td>✓</td>
</tr>
<tr>
<td>Precipitation</td>
<td>✓</td>
</tr>
<tr>
<td>BSA (0.0037%) in PCR</td>
<td></td>
</tr>
<tr>
<td>Sensitivity of detection (cfu/ml)</td>
<td>$10^5$</td>
</tr>
</tbody>
</table>

- Bead beating + Lysis buffer enhanced cell lysis
- BSA improved PCR
Bead-Beater lysis improved extraction of MAP DNA & decreased the detection limit of MAP in milk

* Sensitivity of MAP DNA detection (cfu/ml)

Lysis buffer (LB) + Boil

LB, BB, precipitation

LB, BB, precipitation, BSA

413 bp IS900 product

+ve, -ve controls

Odurum et al. (2001)
Factors that improve PCR–based detection of MAP from milk

• Process ≥ 50 ml milk samples
  – Pre-incubation at 37°C improves recovery of MAP

• Combine the pellet and cream fractions
  – Suspend in 0.75 HPC (hexadecyl pyridinium) to enhance MAP extraction from the cream fraction.

• Lyse in buffer containing 1M Guanidinium thiocyanate

• Use a Bead-beater for optimal MAP cell lysis

• DNA precipitation

• Nested PCR & Bovine serum albumin (BSA, 0.0037%) enhanced the sensitivity of IS900 PCR reactions

➤ can detect 10 or less cfu / ml
II. Screening of Raw & retail milk for MAP

- 133 raw milk samples from 146 individual cows in 14 MAP infected herds. (Gao et al 2009)

**Results:**

- Milk by Culture: 5
- Milk by nested PCR: 23
- Milk by direct PCR: 18

Total: \(N = 133\)
Screening of Raw and retail pasteurized milk for MAP

• 710 locally sourced commercially pasteurized milk samples were screened for MAP

**Results:**

• 15.5% positive by PCR
• **NO** milk samples were culture-positive on Herrold’s egg yolk agar
III. MAP in muscle, lymphatic & organ tissues from cows with advanced JD  
(Mutharia et al 2010)

• Blood, liver, lymph nodes and muscle tissues were collected aseptically from 5 JD-positive cows  
  — Processed fresh, chilled (4C, O/N) or after freezing  
  — Tissues were surface sterilized; samples for culture were cut from within

• MAP was detected using PCR, culture & staining from,  
  — Raw & Cooked tissues, Muscle & humburger patties  
  — Patties contained chopped mesenteric lymph nodes  
  — Meat was cooked to core temperatures of 61°C or ≥71°C

• Sample was homogenized; half decontaminated, MAP recovered for PCR or culture/staining
Results 1

1. No aerobic bacterial counts were detected in raw or cooked samples
   - no fecal contamination of the samples

2. Culture was by MGIT broth (mycobacteria growth indicator tubes) & HEYA (Herrolds egg yolk agar)
   - All MGIT cultures positive for IS900 PCR were also positive for $hspX$ by RT-PC.

3. NO MAP were cultured from blood in all 5 cows

4. HPC decontamination had no significant effect on MAP recovery from tissues
Results cont.

5. High MAP cfu/gm were recovered from intestinal lymph nodes & organs
   \[ \geq 10^3 \text{cfu/g} \] from 7 of 15 livers, mesenteric & ileocaecal lymph nodes; & \textbf{Lower cfu} from 5/15 kidneys, superficial inguinal & prescapular lymph nodes

6. **MAP disseminates to muscle tissues in advanced JD**,  
   - MAP \textit{cultured} from 1 and \textit{detected in 6} of non-decontaminated \textit{raw muscle} samples

5. **MAP can survive cooking**,  
   - MAP were recovered in 1/15 non decontaminated muscle samples cooked to \textbf{61C (medium rare)}  
   - NO MAP were cultured in well done meat (\(\geq 71\))
Conclusion:
1. MAP bacterial were cultured from raw cow milk BUT NOT from commercially pasteurized retail milk
2. MAP DNA was detected in retail pasteurized milk
   - dead bacteria
   - we failed to recover or culture the few MAP present?
3. MAP can survive killing by pasteurization of milk or cooking of beef.
4. Beef should be cooked to ≥ 71C (well done)

Acknowledgement funding from,
Dairy Farmers of Ontario,
Dairy Farmers of Canada,
Alberta Beef Producers,
OMAFRA,
Health Canada, Bureau of Biohazards