AVMA 146th Annual Convention, Seattle, WA
July 11-14, 2009
“Raw Milk Conundrum: The Interplay of Science, Policy and Free Choice”
“Advances in microbiological and molecular assays for assessing raw milk”

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Raw Milk Conundrum
Summarized from “Marler Blog” (www.marlerblog.com)

• **Pros**
  – "Protective effect” against allergies, tooth decay, pathogens (anti-bacterial)
  – Complex microflora (“Hygiene hypothesis”); induced immunity
  – Nutritional and fertility advantages

• **Cons**
  – GI illness: sporadic and outbreak
  – Costs to public health sector, productivity, dairy industry
Raw milk outbreaks

  - 46 outbreaks
  - 1733 illnesses
  - *Campylobacter* (57%), *Salmonella* (26%), *E. coli* O157 (2%), *Staphylococci* (2%)
Chapter 6

Prevalence of *Campylobacter* in the Food and Water supply: Incidence, Outbreaks, Isolation and Detection

William G. Miller and Robert E. Mandrell


### Source (# Cases)

- **Meat/Beef/Pork**: 128 cases, 24 outbreaks
- **Milk/Dairy**: 7,425 cases, 95 outbreaks
- **Miscellaneous**: 1,914 cases, 49 outbreaks
- **Poultry**: 799 cases, 45 outbreaks
- **Produce**: 1,024 cases, 21 outbreaks
- **Seafood/shellfish**: 171 cases, 8 outbreaks
- **Water**: 17,328 cases, 68 outbreaks
- **Unknown**: 2,390 cases

**Total number of outbreaks**: 599

**Total number of outbreaks throughout (Source: 599)**
May-2006, 2\textsuperscript{nd} largest Campylobacter outbreak in US history? (CA prison dairy)

Milk, unpasteurized, 3500 cases, UK-1979

Milk, raw, 500 cases, Switzerland-1981

Milk, unpasteurized, 332 cases, UK-1987

Milk, unpasteurized, 35 cases, Switzerland-1987

Milk, unpasteurized, 110 cases, USA-1988

Milk, heat-treated, >1800 cases, USA-2004

Total 7,425

\*Represents 2 outbreaks.
Minimal Infectious Dose (MID)

- Minimal Infectious Dose depends upon:
  - Virulence of the strain
  - Immune-status of the host and host specificity
  - Complexity of the contaminated sample (food) ingested with pathogen
  - Exposure does not always result in an illness

- **C. jejuni**
  - 500 cells in a single “volunteer” (Robinson, 1981)
  - ~500 cells in volunteers (Black *et al.*, 1988)

- **E. coli O157:H7**
  - 31 to 35 cells, children and adults (Teunis *et al.* 2004)

- Theoretically, milk provides an even distribution of MIDs
Surveillance and outbreaks

- Epidemiology is critical
- Microbiology
  - Isolation of pathogen from complex samples (milk)
    - Enrichment culture
    - ImmunoMagnetic separation (IMS)
    - Selective and/or chromogenic media
    - Subculture of suspect colonies
    - Test many (10-50 cfu) to increase chance of finding outbreak strain (“needle in the haystack”)
  - Genotyping to identify strains
  - Molecular identification without culture (PCR)
Molecular characterization of *Campylobacter jejuni* strains linked to recent milk-related outbreaks and surveillance of California Central Valley dairy environments

Michele Jay, William Miller, Emma Yee, Anna Bates, Paul Rossitto and Robert Mandrell
C. jejuni Outbreak 1

- Correctional facility with on-site dairy (‘Dairy A’)
- Onset dates of May 13–26, 2006
- 1,644 ill inmates/11 facilities
- Pasteurized milk from Dairy A only common food/beverage
- No Campylobacter isolated from milk
- Largest US milk-related Campylobacter outbreak; 2nd largest Campylobacter outbreak ever in US.
Farm Investigation

- Environmental samples were collected between Dec 2006 and Jan 2007
  - Cattle feces
  - Flush alley water
  - Bulk tank raw milk
  - Dairy lagoons
C. jejuni CFU on selective medium: the start of isolation

How many CFU should you pick to find the "needle in the haystack"?
Campylobacter sample collection

Farm Investigation
- Multiple colony picks
  - 6-12 suspect *Campylobacter* from each positive sample
    - 52 isolates confirmed *C. jejuni*
    - Major outer membrane protein (Cmp/MOMP) typing: identified multiple isolates as potential outbreak strain
    - Multilocus sequence typing (MLST) ST-21
MOMP typing (Cmp)

Outer Membrane

Hypervariable loops in OMP

Related strains will have identical DNA sequence for this gene
Methods

MOMP (cmp) typing

- *cmp* gene encodes the *Campylobacter* MOMP
- Sequence polymorphisms make *cmp* typing an epidemiological tool

- Sequence *cmp* gene of large number of environmental strains: identify potential “outbreak strains”
- Further characterize “outbreak strains”
  - PFGE (*Smal*, *KpnI*) and MLST
Sequence variation in hypervariable loops

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<th>Strains</th>
<th>Amino acid sequence</th>
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β13  
L7  
β14  
β15  

N-terminus  
C-terminus  

Diversity (%)  

HV1  HV2  HV3  SV1  HV4  SV2  HV5
Multilocus Sequence Typing (MLST)

- MLST for *Campylobacter* species developed by Dingle et al; JCM, 2001
- 7 housekeeping genes (*aspA, glnA, gltA, glyA, pgm, tkt, and uncA*), ~420 bp each = ~3000 bp total sequence for comparison
- Database: >7800 isolates, ~4000 profiles, time and source
Comparison of Cmp types among C. jejuni
ST-21 strains and other representative isolates

Cmp Typing
Test new genotyping methods in other suspected C. *jejuni* raw milk outbreaks
• *C. jejuni* Outbreak 2
  – 5 cases, Washington state
  – Dec, 2007
  – Linked epidemiologically to consumption of raw milk from “Dairy C”
  – MLST ST-806
• *C. jejuni* Outbreak 3
  - 8 cases, California
  - Onset dates of Nov 23 - Dec 5, 2007
  - All 8 patients reported drinking raw milk/raw chocolate colostrum from “Dairy B”
  - MLST ST-1244
  - PFGE pattern from Dairy B cattle fecal isolates indistinguishable from case-patient isolate
Conclusions

- Isolation of the outbreak strains from dairy environment provides evidence that the source of contamination for each outbreak was at the dairy
- **Cmp typing** provides rapid triaging
- DNA fingerprinting methods (**MLST**) provide higher resolution for screening environmental isolates
- **Cmp + MLST** typing = 8 loci; provides added discrimination
- Persistent and/or predominant strains may exist in the dairy environment
Outbreak 4:
Raw milk suspected, but no isolates available

Only molecular methods
• C. *jejuni* Outbreak 4
  (Karon et al, presented at 2009 ICEID, Atlanta, GA)
  – May-June, 2008
  – Raw milk, cow leasing program
  – 15 cases, California
  – No isolates were saved!
  – 1 patient with Guillain-Barré Syndrome (GBS)
  – 1 sample of 45 day old raw milk was available
  – Opportunity to test detection, limited genotyping and characterization methods
Analysis of milk samples

• Attempts to isolate *C. jejuni* failed
  – Tried enrichment, multiple media, atmospheres
• Milk: DNA purification using multiple kits
• PCR for MLST alleles
• PCR for genes in lipooligosaccharide (LOS) loci
A full aspA (aspartase) allele ("aspA67") was sequenced from PCR products from DNA isolated from raw milk sample

Phylogenetic tree of aspA sequence types

Provided by Bill Miller
The Gram-negative Envelope

- Major outer membrane protein
- O-antigens
- Porin trimer
- Lipopolysaccharide
- Brown's lipoprotein
- Peptido-glycan
- Protein

Gram-negative bacterial endotoxin (lipopolysaccharide, LPS)

O-specific polysaccharide chain
O-specific oligosaccharide subunit
n
Core glycolipid

From: www.bio.davidson.edu/total_membrane.gif
C. jejuni and Guillain-Barre Syndrome (GBS)

- Lipooligosaccharides (LOS) mimic mammalian gangliosides
- Mono-, Di-, Tri-,sialylated glycolipids: GM1a, GM1b, GM2, GD1a, GD1b, GD1c, GD3, etc.

- PCR mapping of LOS genes in DNA from Milk sample
PCR amplification and sequencing of *C. jejuni* LOS genes from total DNA recovered from milk samples

**Amplification**

PCR products for LOS genes *waaV*, *cstIII* and *orf18df*.

- Sequencing of *waaV*
  - Sample 1 – 500 bp 100% identical to a **Class C LOS** gene
  - Sample 2 – 500 bp 100% identical to a **Class D LOS** gene

**Sequencing of cstIII**- a sialyltransferase gene

- Sample 1 – 127 bases 99% identical to a **Class C LOS** gene
Conclusions

• Genetic evidence of *C. jejuni* in raw milk
  – Complete *aspA* sequence (>477 bp)
  – Two *waaV* sequences (500 bp each)
  – 127 bp for *cstIII* (sialyltransferase)

• Evidence of mixed strain sample: two different *waaV* genes (class C and D LOS)

• Patient serum antibodies bind best to LOS of a GBS *C. jejuni* strain
Rapid and Cost-effective Methods for the Detection of Foodborne Pathogens by DNA Microarrays

Photopolymerization: A non-enzymatic signal amplification system

Beatriz Quiñones, WRRC, PSMRU, Albany, CA
Cooperative Agreement with InDevR, Inc., Boulder, CO