A new proactive approach for enhanced microbial risk management and control

CMC Pathogen Reduction Technical Symposium
October 6-7, 2011, Toronto

Shu Chen, Ph.D.

Foodborne Illness in Canada

“An estimated 11 million people suffer food-related illness every year in Canada”.

Listeriosis Outbreak Associated with Ready-to-Eat Meats

The 2008 listeriosis outbreak in Canada:
57 illnesses & 23 death

<table>
<thead>
<tr>
<th>Province</th>
<th>Confirmed Cases</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ontario</td>
<td>41</td>
<td>16</td>
</tr>
<tr>
<td>BC</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Alberta</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Saskatchewan</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Manitoba</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Quebec</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>New Brunswick</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>57</strong></td>
<td><strong>23</strong></td>
</tr>
</tbody>
</table>

http://www.phac-aspc.gc.ca/alert-alerte/listeria/listeria

Salmonella Enteritidis Outbreak Associated with Shell Eggs

From May 1 to November 30, 2010, approximately 1,939 illnesses were reported that are likely to be associated with this outbreak.

www.cdc.gov

360 million eggs recalled!
E. coli O104:H4 Outbreaks Associated with Sprouts

16 countries in Europe and North America had reported 4075 cases and 50 deaths as of 21 July, 2011

<table>
<thead>
<tr>
<th>Country</th>
<th>HUS Cases</th>
<th>HUS Deaths</th>
<th>EHEC Cases</th>
<th>EHEC Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Canada</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Denmark</td>
<td>10</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>France</td>
<td>7</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Germany</td>
<td>857</td>
<td>32</td>
<td>3078*</td>
<td>16</td>
</tr>
<tr>
<td>Greece</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Luxembourg</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Netherlands</td>
<td>4</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Norway</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Poland</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Spain</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Sweden</td>
<td>18</td>
<td>1</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>Switzerland</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>United States of America</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>908</td>
<td>34</td>
<td>3167</td>
<td>16</td>
</tr>
</tbody>
</table>

http://www.euro.who.int/en/

Industry & Government Efforts

- GMP - good manufacturing practices
- Improved technologies to minimize potential pathogens in foods
- HACCP implementation from farm to grocery stores
- Regular plant inspection and food testing
- Safe food handling techniques in homes
- ...
Microbes Have Attitude!

Microbial Strains Have Individuality!

We are different strains of *Listeria monocytogenes*!

Understanding -> Risk assessment - > Control
Salmonella

- 2500+ recognized serovars, most of which share a high level of genetic similarity
- Serotyping is the baseline from which other typing methods are carried out to separate isolates of a specific serovar
- Over 80% of isolated strains belong to top 20 serovars

http://www.about-salmonella.com

L. monocytogenes

• 13 recognized serovars with serovars 1/2a, 1/2b and 4b being the most prevalent in cases of listeriosis
• Different L. monocytogenes strains of a same serovar may exhibit different virulence
Strains of the same species isolated from different sources can be differentiated into subtypes. DNA fingerprinting/sub-typing methods can be used to determine strain relatedness, and identify the potential source of a contamination.

- Knowledge of how pathogens disseminate through the food chain is important in understanding how production practices and food processing contribute to infection.

- The ability to characterize specific strains and determine the primary sources of contamination provides insight into the epidemiology of foodborne pathogens.
PulseNet

The Molecular Subtyping Network for Foodborne Disease Surveillance

PulseNet Canada is coordinated by the Public Health Agency of Canada’s National Microbiology Laboratory (NML) located in Winnipeg, Manitoba.

Participating provincial & federal Laboratories

- Provlab Alberta
- Central Public Health Laboratory (Ontario)
- BC Centre for Disease Control Site
- PHSA Laboratories
- Cadham Provincial Laboratory (Manitoba)
- Queen Elizabeth Hospital (PEI)
- Laboratoire de la santé publique du Quebec
- St. John Regional Hospital
- Newfoundland Public Health Labs
- Saskatchewan Disease Control Laboratory
- Queen Elizabeth II Health Sciences Centre (Nova Scotia)
- Laboratory for Foodborne Zoonoses (PHAC)
- Bureau of Microbial Hazards (Health Canada)
PFGE Example

E. Coli O157:H7 example

|---------------|------------|-----------|------------|------------|----------|---------|-----------|-----------|-----------|------------|

PFGE

Advantages

- “Gold Standard” for subtyping
- DNA restriction patterns are stable & reproducible when the method is highly standardized
- High discriminatory power

Disadvantages

- Time consuming
- Requires high level of skill
- Fragments with the same size may represent different parts of the genome
- Observed PFGE differences may be difficult to interpret
- Some strains are untypeable
Salmonella Enteritidis Outbreak

2-3 weeks from illness to outbreak data

1,813 reported illnesses are likely associated with the outbreak as of October 19, 2010

PulseNet PFGE pattern: JEGX01.0004

The Technology - MLVA

- Bacterial genomes contain tandem duplications of short DNA sequences
  - e.g., ACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACT
  - "tandem repeats"

- The “tandem repeats” vary in “composition”, frequency and copy number between strains

- Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) can be used to determine the identity of a strain
The Technology - MLVA

Isolate 1
ACCACCACCACCACCACC
TGTTGTTGTGTGTGGTT
18 bp

Isolate 2
ACCACCACCACCACCACCACC
TGTTGTTGTGTGTGGTTGG
21 bp

Advantages of MLVA

- **Simplicity** - PCR and fragment sizing
- **Rapid** - Same day result
- **Reproducibility** - Inter-person & inter-lab
- **High discriminatory power** -
  e.g.
  SE highly homogeneous,
  2 PulseNet PFGE patterns
  account for 48% of all SE isolates
  in CDC Database! But MLVA provides
  higher resolution
- **Exchangeability of data** - Portable numeric code
FDA
• Results indicate substantial potential for *Salmonella* to have *persisted in the environment* and to have contaminated eggs.
• Mandatory flock-based SE-control programs that include routine microbiological testing for producers with >50,000 laying hens.
• Measures to prevent SE must be adopted by all egg producers with >3,000 laying hens.

CDC
• Standard subtyping methods alone are not sufficient to determine which reported cases might be outbreak-associated.
• Advanced molecular methodologies are being adopted to help distinguish between outbreak-related cases and sporadic cases.

**Salmonella Surveillance**

• Analysis of Environmental Samples

• Routine testing of > 480 Pullet (0-19 weeks) and Layer (19-72 weeks) operations across Ontario

• 5 samples taken from each farm environment at 10 and 60 weeks of age

• Sample sites: Floors, Walls, Fans, Egg Belts, Manure

• Other sites sampled infrequently
**Salmonella Isolation**

**Immunomagnetic Separation: MFLP-84**

15.6% higher sensitivity than standard culture method MFHPB-20 (Lynch et al. 2004).

---

**Salmonella Characterization**

Analyses performed 2009-2010

Target: 10,000 samples

8,377 samples tested (Sept 2010)

1,403 *Salmonella* spp. isolates (16.74%)

19 *S. Enteritidis* isolates (1.35%)

All isolates frozen preserved at -80°C in 10% glycerol for further analysis:

**MLVA**

- 02-03-05-12-21-11
- 03-03-08-12-13-04
- 03-12-04-10-10-05

**Serotyping - Phagetyping**

**Antimicrobial resistance**
Tracking *Salmonella* Enteritidis on Poultry Farms

**Sample sources:**
- Different Farms: A, B, C
- Different Barns: 1, 2, 3, 4
- Different Dates: Feb. 25 – Nov. 23, 2009
- Different Sites:
  - Floors
  - Walls
  - Cages
  - Egg belt
  - Nest box
  - Fan
  - Litter
  - Manure

<table>
<thead>
<tr>
<th>FARM</th>
<th>Sampling Date (2009)</th>
<th>Sampling Site</th>
<th>Sampling Barn No.</th>
<th>DNA Fingerprint Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>FARM A</td>
<td>February 25</td>
<td>Floors</td>
<td>BARN 1</td>
<td>01-03-03-02-10-00</td>
</tr>
<tr>
<td>FARM A</td>
<td>April 21</td>
<td>Floors</td>
<td>BARN 1</td>
<td>01-03-03-02-10-00</td>
</tr>
<tr>
<td>FARM A</td>
<td>November 23</td>
<td>Litter</td>
<td>BARN 2</td>
<td>01-02-03-02-10-00</td>
</tr>
<tr>
<td>FARM A</td>
<td>November 23</td>
<td>Walls</td>
<td>BARN 2</td>
<td>01-02-03-02-10-00</td>
</tr>
<tr>
<td>FARM A</td>
<td>November 23</td>
<td>Fans</td>
<td>BARN 2</td>
<td>01-02-03-02-10-00</td>
</tr>
<tr>
<td>FARM A</td>
<td>November 23</td>
<td>Nest Boxes</td>
<td>BARN 2</td>
<td>01-02-03-02-10-00</td>
</tr>
<tr>
<td>FARM A</td>
<td>November 23</td>
<td>Egg Belts</td>
<td>BARN 2</td>
<td>01-02-03-02-10-00</td>
</tr>
</tbody>
</table>

- Different farms carried different strains
- Different barns of the same farm had different strains
- Farm/barn-specific strains were re-isolated at a later date
**Toward “Real-Time” Surveillance**

*Monitor trends*

*Index*

- **Resident or transient?**
  - Population-dynamics

- **New or emerging?**
  - Virulence?, Resistance?

Subtype data can be used to facilitate surveillance of *Salmonella* in the poultry environment

---

**MLVA Database for *L. monocytogenes***

**Main goal** - to provide an avenue to allow implementation of proactive and cost-effective monitoring/prevention approaches to prevent/minimize food-borne listeriosis

**Objectives:**

- to index a large collection of *L. monocytogenes* strains isolated from Ontario’s food chain in last 15 years and from Ontario’s historical clinical cases using MLVA analysis

- to identify predominant and persistent types and to assess potential risks associated with the predominant and persistent types

- to establish a database which can be used as a part of future routine monitoring and surveillance programs for systematic risk assessment, management and control
**L. monocytogenes Strains**

- **300+** – OAHPP Ontario clinical cases
- **50+** – HC Validation & control
- **2000+** – UofG Ontario food & agriculture
- **100+** – CFIA Imported food

Indexing All Strains by MLVA Analysis

---

**L. monocytogenes Strain Analysis**

- **300** – OAHPP PFGE
- **200** – PHAC CGF
- **2500** – UofG MLVA

PFGE - Pulsed-field gel electrophoresis
CGF - Comparative genomic fingerprinting

- **2-3** – CFIA WGS

WGS - Whole genome sequencing
MLVA Method for *L. monocytogenes*

Evaluated 26 primer pairs – selected eight specific VNTR loci
Established & optimized two multiplex PCR reactions

**L. monocytogenes Strains - Validation**

<table>
<thead>
<tr>
<th>No of Strains</th>
<th>Strain Source</th>
<th>No of Strains</th>
<th>Strain Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CHEESE 1</td>
<td>1</td>
<td>SPORADIC: USA</td>
</tr>
<tr>
<td>2</td>
<td>MEAT 1</td>
<td>1</td>
<td>SAUSAGE</td>
</tr>
<tr>
<td>1</td>
<td>CHEESE 2</td>
<td>1</td>
<td>PLACENTA</td>
</tr>
<tr>
<td>1</td>
<td>TURKEY FRANK</td>
<td>1</td>
<td>CUCUMBER</td>
</tr>
<tr>
<td>2</td>
<td>RAW MILK</td>
<td>1</td>
<td>FETA CHEESE</td>
</tr>
<tr>
<td>1</td>
<td>MEAT 2</td>
<td>4</td>
<td>OUTBREAK I</td>
</tr>
<tr>
<td>1</td>
<td>ICE CREAM</td>
<td>1</td>
<td>ATCC 19115</td>
</tr>
<tr>
<td>1</td>
<td>SPORADIC, Canada</td>
<td>2</td>
<td>LIQUOR</td>
</tr>
<tr>
<td>1</td>
<td>CHEESE</td>
<td>3</td>
<td>OUTBREAK II</td>
</tr>
<tr>
<td>2</td>
<td>BEEF</td>
<td>1</td>
<td>FOODBORNE</td>
</tr>
<tr>
<td>1</td>
<td>RAW PORK</td>
<td>1</td>
<td>COLESLAW-OUTBREAK</td>
</tr>
<tr>
<td>1</td>
<td>SALAMI</td>
<td>1</td>
<td>CHEESE</td>
</tr>
<tr>
<td>1</td>
<td>ATCC 7644</td>
<td>1</td>
<td>RAW MILK</td>
</tr>
<tr>
<td>1</td>
<td>FAECES</td>
<td>1</td>
<td>RAW BEEF</td>
</tr>
<tr>
<td>1</td>
<td>CHEESE</td>
<td>1</td>
<td>CHICKEN</td>
</tr>
<tr>
<td>1</td>
<td>SALAMI</td>
<td>1</td>
<td>OUTBREAK, Canada</td>
</tr>
<tr>
<td>1</td>
<td>ATCC 19113</td>
<td>1</td>
<td>SHEEP</td>
</tr>
<tr>
<td>2</td>
<td>BEEF</td>
<td>1</td>
<td>CHICKEN</td>
</tr>
</tbody>
</table>
L. monocytogenes Strain Relatedness

Matching the Fingerprints/Codes for L. monocytogenes
Results So Far

- The MLVA method has similar or better discrimination power than PFGE

- Approximately 2400 *L. monocytogenes* strains from Ontario’s agriculture & food chain and clinical cases has been subtyped using MLVA

- Preliminary data analysis identified distinct clusters, predominant strains, many links among the strains & potentially persistent strains in our food system

- Efforts are underway to complete data analysis to determine potential epidemiological links among strains, and identify predominant and persistent *L. monocytogenes* genotypes in Ontario’s food and health chain
Direct Typing of *L. monocytogenes* in Food Samples

**Pure Culture**

**In Food Mix (5% target DNA + 95% DNA from pre-enriched food broth)**

Food Mix = Sausage, Milk, Ham, Smoked Chicken & Lettuce

Results remained the same in the presence of food mix

<table>
<thead>
<tr>
<th>Food type (serotype, strain)</th>
<th>No. of samples typed correctly/total no. of samples tested (CFU/25 g or mL- 48 hrs in LEB)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Egg salad (4b, coleslaw/outbreak)</td>
<td>0/2</td>
</tr>
<tr>
<td>Vegetable mix (4b, coleslaw/outbreak)</td>
<td>0/2</td>
</tr>
<tr>
<td>Soft cheese (4b, cheese)</td>
<td>0/2</td>
</tr>
<tr>
<td>Beef roast (4b, cheese)</td>
<td>0/2</td>
</tr>
<tr>
<td>Smoked salmon (1/2b, salami)</td>
<td>0/2</td>
</tr>
</tbody>
</table>

Direct Typing of *L. monocytogenes* in Artificially Contaminated Food Samples
### Detection/typing of *L. monocytogenes* in post-spiked pre-enriched food broth

<table>
<thead>
<tr>
<th>Food type</th>
<th>No. of samples typed correctly/ total number of samples tested</th>
<th>CFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Sausage (beef and pork)</td>
<td>0/5 0/6 4/6 6/6</td>
<td></td>
</tr>
<tr>
<td>Homogenized milk</td>
<td>0/5 0/6 6/6 6/6</td>
<td></td>
</tr>
<tr>
<td>Cooked Ham</td>
<td>0/5 0/6 6/6 6/6</td>
<td></td>
</tr>
<tr>
<td>Smoked Chicken</td>
<td>0/5 0/6 4/6 6/6</td>
<td></td>
</tr>
<tr>
<td>Lettuce</td>
<td>0/5 0/6 5/6 6/6</td>
<td></td>
</tr>
</tbody>
</table>

### LOD (CFU/mL) of different serotypes

<table>
<thead>
<tr>
<th>Food type</th>
<th>LOD (CFU/mL) of different serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>4b (ATCC 19115)</strong></td>
</tr>
<tr>
<td>Sausage (beef and pork)</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Homogenized milk</td>
<td>$10^3$</td>
</tr>
<tr>
<td>Cooked Ham</td>
<td>$10^3$</td>
</tr>
<tr>
<td>Smoked Chicken</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Lettuce</td>
<td>$10^4$</td>
</tr>
</tbody>
</table>

Toward “Real-Time” Tracking

1. Pre-enrichment & DNA extraction
2. PCR “-”
3. PCR “+” Typing
4. Data analysis (compare with database)
5. MLVA fingerprinting

Toward Proactive Strain Surveillance

- Farm 1
- Farm 2
- Farm 3
- Farm 4
- Farm 5
- Importer 1
- Importer 2
- Importer 3
- Importer 4
- Processed plant
- Workers
- Food Processing Equipment
- Finished Products
**Pathogen:** *Campylobacter*, *Salmonella*, *E. coli* O157:H7, *Listeria monocytogenes*…
*occur less often, direct detection*

**Indicator:** *E. coli*, *Listeria*…
*occur more often, more assurance (?)*

**Objectives:**

1) Identify points of concern from growth to packing, on leafy green and fresh market tomato farms
2) Link environmental sources of contamination with contaminated produce samples
Pin-pointing Contamination on Leafy Green and Fresh Market Tomato Farms

Methodology:
- Extensive, tailored sampling plan on 4 different farms:
  - Fresh market tomato, parsley, spinach and romaine
- Samples
  - Mid-growth: Irrigation water, soil and crops
  - Pre-harvest: Irrigation water, soil and crops
  - At harvest: crops, hand swabs, harvest equipment swabs
- Processing:
  - Hydro-cooling: Ice
  - Washing/cooling: wash water, crops
  - Packing: hand swabs, crops
- All samples analyzed for *E. coli*
- *E. coli* isolates genotyped using Amplified Fragment Length Polymorphism (AFLP) to identify contamination sources


Lettuce Farm Pilot Study

*E. coli* test outcomes:
- 34 *E. coli* positive samples (433 samples tested)
- 309 *E. coli* isolates fingerprinted
- 91 fingerprint types

Irrigation water and soil were the potential sources of *E. coli* contamination for the Romaine lettuce.

Recommendations (for farmer)

- Know the water source and its typical *E. coli* range: stream, river, with variable quality?
- Test irrigation water and limit risks by extending time between irrigation and harvest
- Create and implement a water sanitation program for post-harvest water


Tracking Sources BEFORE Outbreaks

Listeria
Salmonella
Campylobacter
*E. coli* O157:H7

Source?
Toward Proactive Strain Surveillance

- Know what pathogen strains are around particular operations
  - Suppliers, ingredients
  - Biofilms on equipment
  - Employee
  - Production facility environment
  - Resident or transient
  - Other
- Implement a rapid and effective plan in response to a positive event
- Practice based on targeted effective control and elimination processes

Proactive Strain Surveillance

- Obtain information beyond “YES/NO” qualitative results
- Provide early warning to reduce risks of recalls and outbreaks
- Act proactively beyond “plant floor inspection” and “test and hold” protocols
- Improve and verify HACCP
- Protect consumers more effectively
- Enhance consumer/customer trust
Concluding Remarks

- We strongly believe that molecular typing (strain surveillance) is the way forward and MLVA is a step in that direction.

- MLVA may be implemented as a discriminating and epidemiologically relevant subtyping method for ongoing surveillance.

- The full synergy of combining molecular typing data and routine surveillance information and interpreting them jointly can contribute to improving and better targeting existing control measures.

- The introduction of this approach will require more open discussions, understanding and corporation among all stakeholders involved.

Acknowledgements

- Saleema Saleh-Lakha, Jiping Li, Carlos Leon-Velarde, Susan Lee & Anli Gao (Laboratory Services, UofG)
- Vanessa Allen (OAHPP)
- Franco Pagotto (Health Canada)
- Eduardo Taboada & Roger Johnson (PHAC)
- Joseph Odumeru (MOE)
- Dele Ogunremi & Burton Blais (CFIA)
- Gavin Downing, Lindsay Arthur, Moustapha Oke (OMAFRA)
- Pam Bolton & Albert Visser (Egg Farmers of Ontario)

- Staff at AFL, Lab Services & Toronto Public Health Labs

- Funding from OMAFRA Food Safety Research Program & Poultry Industry Council (PIC)