Advances in Rapid Microbiology Testing Methods

Eric W. Brown, Ph.D.

Acting Director, Division of Microbiology
Office of Regulatory Science, Center for Food Safety & Applied Nutrition
U.S. Food & Drug Administration

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## Major Outbreaks Over the Last 3 Years

<table>
<thead>
<tr>
<th>YEAR</th>
<th>AGENT</th>
<th>VEHICLE</th>
<th>CASES</th>
<th>SOURCE</th>
<th>STATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>Salmonella Saintpaul</td>
<td>jalapeno/serrano peppers</td>
<td>1442**</td>
<td>Mexico</td>
<td>ML</td>
</tr>
<tr>
<td>2008</td>
<td>Salmonella Typhimurium</td>
<td>Peanut butter/peanut paste</td>
<td>677</td>
<td>GA</td>
<td>ML</td>
</tr>
<tr>
<td>2006</td>
<td>Salmonella Tennessee</td>
<td>peanut butter</td>
<td>628</td>
<td>GA</td>
<td>ML</td>
</tr>
<tr>
<td>2009</td>
<td>*Salmonella Saintpaul</td>
<td>Sprouts</td>
<td>235</td>
<td></td>
<td>ML</td>
</tr>
<tr>
<td>2006</td>
<td>E coli O157:H7</td>
<td>spinach</td>
<td>204</td>
<td>CA</td>
<td>ML</td>
</tr>
<tr>
<td>2006</td>
<td>Salmonella Typhimurium</td>
<td>tomatoes</td>
<td>190</td>
<td>OH</td>
<td>ML</td>
</tr>
<tr>
<td>2009</td>
<td>Bacillus Cereus</td>
<td>Macaroni and cheese</td>
<td>150</td>
<td>CT</td>
<td>ML</td>
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<tr>
<td>2009</td>
<td>*Salmonella Typhimurium</td>
<td>Cucumber (suspected*)</td>
<td>118</td>
<td></td>
<td>ML</td>
</tr>
<tr>
<td>2006</td>
<td>Salmonella Newport</td>
<td>tomatoes</td>
<td>107</td>
<td>VA</td>
<td>ML</td>
</tr>
<tr>
<td>2006</td>
<td>E coli O157:H7</td>
<td>lettuce</td>
<td>81</td>
<td>CA</td>
<td>ML</td>
</tr>
</tbody>
</table>
Serotypes found during Saintpaul pepper investigation - 2008

- Abaetetuba
- Albuquerque
- Anatum
- Braenderup
- Cerro
- Derby
- Give
- Huvudsta
- Infantis
- Javiana
- Koumra
- Madelia
- Mbandaka
- Michigan
- Minnesota

- Muenchen
- Newport
- Ohio
- Oranienburg
- Pomona
- Saintpaul
- Sandiego
- Soahanina
- Tallahassee
- Tucson
- Typhimurium
- Weltevreden
- S. enterica subsp. arizonae
- S. enterica subsp. diarizonae
- S. enterica subsp. houtenae
## Additional Significant Outbreaks (last 3 years)

<table>
<thead>
<tr>
<th>Year</th>
<th>Pathogen</th>
<th>Food or Product</th>
<th>Cases</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td><em>Salmonella</em> Orianburg</td>
<td>Round tomatoes</td>
<td>79</td>
<td>ML</td>
</tr>
<tr>
<td>2009</td>
<td>E. Coli 0157:H7</td>
<td>Cookie Dough</td>
<td>77</td>
<td>ML</td>
</tr>
<tr>
<td>2009</td>
<td><em>Salmonella</em> Rissen</td>
<td>White Pepper Spice (suspected)</td>
<td>72</td>
<td>ML</td>
</tr>
<tr>
<td>2009</td>
<td><em>Salmonella</em> Newport</td>
<td>Unknown</td>
<td>72</td>
<td>ML</td>
</tr>
<tr>
<td>2006</td>
<td>E coli O157:H7</td>
<td>lettuce</td>
<td>71</td>
<td>CA</td>
</tr>
<tr>
<td>2007</td>
<td><em>Salmonella</em> Wandsworth</td>
<td>veggie booty</td>
<td>69</td>
<td>China/Egypt</td>
</tr>
<tr>
<td>2007</td>
<td><em>Salmonella</em> Newport</td>
<td>tomatoes</td>
<td>65</td>
<td>VA</td>
</tr>
<tr>
<td>2008</td>
<td><em>Salmonella</em> Litchfield</td>
<td>cantaloupe</td>
<td>57</td>
<td>Honduras</td>
</tr>
</tbody>
</table>
Recent Outbreaks
- Part of an increasing trend

• There are several possible explanations for the apparent increase
  – Better and more rapid detection of outbreaks
  – Increase in sale of fresh-cut produce
  – Wider distribution from a more limited production area
  – Globalization of the produce supply
  – Increase in the numbers of consumers at high risk for foodborne illnesses
We Need Rapid Methods

• Increase number of samples analyzed for routine surveillance of domestic and imported products

• Tracking/identifying sources of outbreaks (molecular epidemiology)

• Problem 1: Improved sampling and recovery of contaminants

• Problem 2: Rapid fingerprinting of micro-organisms or identification of new chemical toxins
Why do we need rapid methods?

Clinical ID and fingerprint

Identify Food and confirm Fingerprint

Product enters commerce

Number of cases

Days
Microbiological investigations of Salmonella are a 3-step process:

1. **Detection (species)**
   - Is it *Salmonella*?

2. **Identification (serotype)**
   - What kind of *Salmonella* is it?

3. **Traceback (subtype)**
   - Is it the outbreak strain?

Mitigation strategies include:

- Any method that combines two of the three boxes—particularly boxes I and II.

- Any method(s) that shrink the width (i.e., timespan) of one or more boxes.
“The Smokin’ Jalapeno”

(Firm ran out of plastic cover for bedding-Sample collected scored positive for *Salmonella* Saintpaul)
Another Kind of ‘Risk Assessment’
• Indigenous microbial population on produce - $10^2$ to $10^{10}$ cfu/g
Needle in the Haystack
- Escherichia coli 0157:H7
- STEC
- Listeria
- Salmonella
- Shigella
- Enterobacter (Chronobacter) sakazakii
- Fungi
- Staphylococcus aureus (enterotoxin)
- Clostridium (toxins)
- Francisella tularensis
- Bacillus anthracis, B. cereus
An Improved Soak Method for Canteloupes suspect for Salmonella

Cantaloupe in bag with preenrichment broth

- Shake, 100 rpm 5 min
- Rinse (25 mL)

Pre-enrichment

- Soak
- 24 h, 35°C

Selective enrichment

- 24 h, 35°C and 42°C

Selective plating

- 24 h, 35°C and 42°C
Incubate 6 hrs @ 37°C

25 g

225 ml H₂O/BPW

10 ml

Centrifuge 5000 rpm for 10 min
Decant supernatant
Resuspend pellet in 100 ul H₂O/BPW

Plate on Chromogenic Agar

(concurrently)
continue PIF/H₂O Incubation O/N at 37°C

Confirm with RT-PCR, RapidID 32 E, Class Fatty Acid Analysis
Molecular Detection Methods
Conventional and Real-Time PCR allows us to target specific bacterial pathogens despite the background microbial load
Detection of Live *Salmonella* sp. Cells in Produce by a TaqMan-Based Quantitative Reverse Transcriptase Real-Time PCR Targeting *invA* mRNA


Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland

Received 24 November 2008/Accepted 3 April 2009

*Salmonella enterica* contamination in foods is a significant concern for public health. When DNA detection methods are used for analysis of foods, one of the major concerns is false-positive results from the detection of dead cells. To circumvent this crucial issue, a TaqMan quantitative real-time RT-PCR (qRT-PCR) assay with an RNA internal control was developed. *invA* RNA standards were used to determine the detection limit of this assay as well as to determine *invA* mRNA levels in mid-exponential-, late-exponential-, and stationary-phase cells. This assay has a detection limit of 40 copies of *invA* mRNA per reaction. The levels of *invA* mRNA in mid-exponential-, late-exponential-, and stationary-phase *S. enterica* cells was approximately 1 copy per 3 CFU, 1 copy per CFU, and 4 copies per 10^4 CFU, respectively. Spinach, tomatoes, jalapeno peppers, and serrano peppers were artificially contaminated with four different *Salmonella* serovars at levels of 10^5 and less than 10 CFU. These foods were analyzed with qRT-PCR and with the FDA’s Bacteriological Analytical Manual *Salmonella* culture method (W. A. Andrews and T. S. Hammack, in G. J. Jackson et al., ed., Bacteriological analytical manual online, http://www.cfsan.fda.gov/~ebam/bam-5.html, 2007). Comparable results were obtained by both methods. Only live *Salmonella* cells could be detected by this qRT-PCR assay, thus avoiding the dangers of false-positive results from nonviable cells. False negatives (inhibition of the PCR) were also ruled out through the use of an RNA internal control. This assay allows for the fast and accurate detection of viable *Salmonella* spp. in spinach, tomatoes, and in both jalapeno and serrano peppers.

*Salmonella enterica* contamination in various foods is a significant public health concern, domestically and internationally (22, 29, 37). *Salmonella* infects millions of people every year, accounting for an estimated 9.7%, 25.6%, and 30.6% of illnesses, hospitalizations, and deaths, respectively, of the total U.S. food-borne diseases caused by known food-borne pathogens (29). Consumption of fresh fruits and produce increased almost 50% between 1970 and 1994 (28). Fresh produce is exists for the development of faster culture-independent screening and detection methods for this pathogen in produce.

In recent years, a plethora of new molecular methods based on *Salmonella* DNA detection (e.g., *invA* gene) either by conventional or real-time PCR have been developed (23, 27, 41). Real-time PCR (quantitative PCR [qPCR]) is faster and more sensitive than conventional PCR and provides real-time data, with a low false-positive rate [qPCR (28, 30)].
Microbiological investigations of Salmonella are a 3-step process:

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3. **Traceback (subtype)**

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Identification of major *Salmonella* serotypes - CDC

Bioplex – based serological identification

**CDC Molecular Serotyping Protocol**

Molecular serotyping using xMAP

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**Salmonella Typhimurium**

O antigen $\rightarrow$ LPS

Flagella $\rightarrow$ H antigen
X-MAP TECHNOLOGY

BIOPLEX/LUMINEX PLATFORM
New Frontiers in Molecular Epidemiology

Sample throughput

Tests per Sample

- microarray
- multiplex RT-PCR
- northern blot
- Mass Spec
- Real-Time PCR
- xMAP Technology
RT-PCR mini-array
Portable and rapid (<2 hrs)
Digital signal processing
Traditional qPCR chemistry

PREMITEST
Microbiological investigations of *Salmonella* are a 3-step process:

1. **Is it *Salmonella***?
   - **Detection (species)**
   - *I*  

2. **What kind of *Salmonella* is it?**
   - **Identification (serotype)**
   - *II*  

3. **Is it the outbreak strain?**
   - **Traceback (subtype)**
   - *III*  

Mitigation strategies include:

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The National Molecular Subtyping Network for Foodborne Disease Surveillance

West   Mountain   South Central   North Central   Midwest   Mid-Atlantic   Southeast   Northeast

COLORADO   OREGON   CALIFORNIA   NEVADA   IDAHO   UTAH   ARIZONA   MONTANA   WYOMING

WASHINGTON   NEW MEXICO   MONTANA   NORTH DAKOTA   MINNESOTA   WISCONSIN

WYOMING   WASHINGTON   IDAHO   MONTANA   NORTH DAKOTA   MINNESOTA

COLORADO   WYOMING   MONTANA   NORTH DAKOTA   MINNESOTA   WISCONSIN

FDA-CFSAN   FDA-ORA   FDA-ORA   FDA-ORA   FDA-ORA   FDA-ORA   FDA-ORA   FDA-ORA

Area Laboratories
PulseNet Central
County/City Laboratories
USDA Laboratories
FDA Laboratories

West  Mountain  South Central  North Central  Midwest  Mid-Atlantic  Southeast  Northeast
May 2005 S. Enteritidis food-related clusters

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Location</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>JEGX01.0004</td>
<td>NC and VT (eggs?)</td>
<td></td>
</tr>
<tr>
<td>JEGX01.0005</td>
<td>UT food</td>
<td></td>
</tr>
<tr>
<td>JEGX01.0018</td>
<td>GA hospital (eggs)</td>
<td></td>
</tr>
<tr>
<td>JEGX01.0021</td>
<td>SC buffet restaurant (eggs)</td>
<td></td>
</tr>
<tr>
<td>JEGX01.0056</td>
<td>GA hospital (eggs)</td>
<td></td>
</tr>
</tbody>
</table>

Pattern JEGX01.0004 makes up 40% of all of the S. Enteritidis seen in the PulseNet database.
Three very common S. Enteritidis patterns that are difficult to differentiate. They are often found together or in concurrent outbreaks.

Difference is in a band-shift at ~40 kb

JEGX01.0018
JEGX01.0021
JEGX01.0056
“Simultaneous analysis of combined enzymes provides insight into sources and lineages of epidemic clones of *Salmonella Typhimurium*”
Optical Mapping

Courtesy of Dr.
Michael Kotewicz
CFSAN-OARSA

Multiple contigs; assembly

Linear representation of bacterial genome “consensus map”
Optical Mapping

Peanut paste strain SL871 chromosomal markers 1-7
(PFGE type 0459, contains genes from plasmid R721)

Markers

SL871 R721+ [Ncol]

1

2

LT2 [Ncol] (in silico)

Gifsy-2 [Ncol] (in silico)

LT2.. [Ncol] (in silico)

962Mb Fels-1 [Ncol] (in silico)

R721_2527 [Ncol] (in silico)
Optical Mapping: Using the right tool at the right time;

Insertions and deletions
Whole Genome Sequencing

454 Technologies whole-genome sequencing systems:

- allows for multiple coverage of a complete bacterial pathogen’s genome in 3 days
- provides raw data for SNP discovery
- allows for identification of rapidly changing genetic markers

From where and how are genotypic targets derived?
New instrumentation installed in Fall 2009

Ability to collect 4 microbial genomes per day.

Charlie Wang
Genome scanning strategies:

Currently, the whole-genome analyses of 60 additional *Salmonella* genomes are underway.
Genome scanning strategies:

Bioinformatic tools are now under development to meet these data density tools including alignment tools, search engines, and genome assembly software.

Dr. Kurt Lienau
Phenotypic typing strategies:

(1) Phenotype Microarray

(2) LC-MS Protein Profiling

(3) Antibiotic Resistance Profiling ~NARMS
Microbiological investigations of Salmonella are a 3-step process:

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Objectives of Ibis T5000 Biosensor Technology

- Broad identification of all microbes
  - Bacteria, Viruses, Fungi, Protozoa
  - No culturing
  - No DNA sequencing

- Mixed populations of microbes
  - Quantitative

- High resolution genotyping, strain identification and antibiotic resistance determination

- Emerging infectious disease

- Cost effective, rapid, high throughput
Sample Collection and Processing

**Sample Collection**
6-24 hour indoor and outdoor air samples

**Sample Preparation**
Sample lysis followed by DNA/RNA purification

**Sample and Enzyme added to Ibis assay plate**
- Ibis plate
- Enzyme
- PE Janus robot or hand pipetting

**PCR**

**Equipment**
- KingFisher
- Eppendorf
FDAs Rules of the Game...

...Mastering the 4 R’s of the Microbial Detection/Identification Method

**Robustness:** The technique must reveal enough genetic differences to effectively discriminate between closely related strains

**Repeatability:** The technique must be reliable enough to consistently yield the same result across laboratories and at different times

**Rapidity:** The technique must be rapid enough to stay in tune with an investigation

**Recognition:** The technique must be recognized in court as a ‘tried and true’ method for science-based regulatory investigations
Summary

• FDA is facing new analytical challenges as a result of the globalization of the food supply and changes in many other areas, including consumer preferences and food industry practices
  – Inexpensive rapid screening tools
  – Faster, selective sample cleanup and enrichment
  – Multi-analyte and/or multi-pathogen detectors
  – Broad range, non-targeted chemical and biochemical detectors

• Protecting and promoting the public health remains our focus.
1942

- *Staphylococcus aureus*
- *Salmonella*
- *Clostridium botulinum*
- *Streptococci*
Emergence of Foodborne Pathogens

- Campylobacter jejuni
- Clostridium botulinum (infant)
- E. coli 0157:H7
- Listeria monocytogenes
- Salmonella Enteritidis
- Vibrio cholerae (Latin America)
- Vibrio vulnificus
- STECs
- 4,5,12:i:- Salmonella
- Yersinia enterocolitica
- Norwalk and Norwalk-like viruses
- Rotavirus
- Cryptosporidium parvum
- Giardia lamblia
- Toxoplasma gondii
- Bovine spongiform encephalopathy prion
- V. Parahaemolyticus
- E. sakazakii
- MRSA
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