Development of an FDA/AOAC Standard Method for Detection of *Cronobacter*

Keith A. Lampel, Ph.D.
Director, Division of Microbiology
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Since 1906...

The Dining Room of "The Poison Squad"
Safe, Wholesome, Sanitary Foods
Federal Food, Drug, and Cosmetic Act

- **Sec. 402**: A food shall be deemed to be adulterated-
  - (a) (1) if it bears or contains any *poisonous or deleterious* substance which may render it injurious to health; ....
  - (3) if it consists in whole or in part of any filthy, putrid, or decomposed substance, or if it is otherwise unfit for food; or
  - (4) if it has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health; or (5) diseased animal.. or (6) if its container...of any *poisonous or deleterious* substance....
Foodborne Illnesses

- 76 million illnesses in the US
- 325,000 hospitalizations
- 5,200 deaths
- Known pathogens account for an estimated 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths annually
Cases of *E. sakazakii* disease among infants

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>No. cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1961</td>
<td>England</td>
<td>2</td>
</tr>
<tr>
<td>1965</td>
<td>Denmark</td>
<td>1</td>
</tr>
<tr>
<td>1979</td>
<td>Georgia (USA)</td>
<td>1</td>
</tr>
<tr>
<td>1982</td>
<td>Indiana, Oklahoma</td>
<td>1 each</td>
</tr>
<tr>
<td>1983</td>
<td>Netherlands</td>
<td>8</td>
</tr>
<tr>
<td>1985</td>
<td>Missouri (USA)</td>
<td>1</td>
</tr>
<tr>
<td>1988</td>
<td>USA</td>
<td>2</td>
</tr>
<tr>
<td>1989</td>
<td>Portugal, Iceland, Tennessee</td>
<td>1, 3, 3</td>
</tr>
<tr>
<td>1990</td>
<td>Maryland (USA)</td>
<td>1</td>
</tr>
<tr>
<td>1991</td>
<td>Ohio (USA)</td>
<td>1</td>
</tr>
<tr>
<td>2000</td>
<td>North Carolina (USA)</td>
<td>1</td>
</tr>
<tr>
<td>2001</td>
<td>Israel, Belgium, Tennessee (USA)</td>
<td>2, 1, 1</td>
</tr>
<tr>
<td>2002</td>
<td>Israel, Wisconsin (USA)</td>
<td>2, 1</td>
</tr>
<tr>
<td>2003</td>
<td>USA</td>
<td>6</td>
</tr>
<tr>
<td>2004</td>
<td>France, USA</td>
<td>2, 2</td>
</tr>
<tr>
<td>2005</td>
<td>USA</td>
<td>2</td>
</tr>
</tbody>
</table>

Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 12, No. 8, August 2006
Sample size and preparation
Needle in the Haystack
Finding that needle

Bacteriological and molecular
In the spring of 2002, FDA issued U.S. Health Care Alert for risk of *E. sakazakii* infection associated with the consumption by neonates of milk-based powdered infant formulas and fortifiers.

**Intrinsic contamination:** ingredients that constitute powdered infant formula or the processing environment may be a source of *E. sakazakii*
Research to Develop Detection Methods

E. sakazakii in powdered infant formula
Day 1: Make an isolation streak and spread plate from each EE broth onto VRBG Agar.

Day 2: Pick 5 presumptive positive colonies - streak onto TSA and check for yellow pigment production.

Day 3: Yellow colonies are confirmed with the API 20E test kit.

Pre-Enrichment (O/N)

Enrichment (O/N)

Selection (O/N)

Confirmation (O/N)

Current FDA Protocol

1 g 9 ml Sterile Warm H₂O
10 g 90 ml Sterile Warm H₂O
100 g 900 ml Sterile Warm H₂O

Incubate Overnight at 36ºC

Incubate Overnight at 36ºC

Incubate Overnight at 36ºC

Incubate Overnight at 25ºC

Day 1

Day 2

Day 3

Day 4

Day 5
Our GOAL was to:

...develop a sensitive, robust, and rapid method for detection of *Enterobacter sakazakii* in powdered infant formula.

Improving upon:
1. More speedy and consistent enrichment
2. Detection with PCR-assay and chromogenic agar
3. Overall simplicity of reagents and protocols
Revised Method

PIF

300 g

2.7 liters BPW

Incubate 6 hrs @ 37°C

Centrifuge 3000 x g for 10 min
Decant supernatant
Resuspend pellet in 200 µl H₂O

Plate on agar plates

Incubate overnight @ 37°C

concurrently, continue incubation 18-24 hr at 37°C

Confirm with RT-PCR, API 20E, RapidID 32 E
AOAC

• International effort in method development
• Pre-collaborative study
  – Inclusive and exclusive strain list
  – samples
• Current status of FDA method-submitted and reviewed
• Validation-FDA-BAM
Druggan-Forsythe-Iversen Agar (DFI)

5-bromo-4-chloro-3-indolyl-alpha,D-glucopyranoside (XalphaGlc) → alpha-glucosidase → indigo pigment (blue-green)

Secondary: hydrogen sulfide production

Iversen et al., 2005
R&F Agar

5-bromo-4-chloro-3-indoxyl-alpha-D-glucopyranoside and 5-bromo-4-chloro-3-indoxyl-beta-D-cellobioside

alpha-glucosidase

Blue black or green?

Secondary: fermentation of D-arabitol, adonitol, and sorbitol
Taqman Real-Time PCR

Target: dnaG on the macromolecular synthesis operon (Seo et al., 2004)

- a. Taqman Universal PCR master mix with ABI Prism 7000 Sequence Detection System
  - 2 × Taqman Master mix: 25 µl
  - Forward primer: 5 µl (900 nM) / Reverse primer: 5 µl (900 nM) / probe: 5 µl (250 nM)
  - H₂O: 5 µl / DNA template: 5 µl / Final volume: 50 µl
  - 95 °C for 10 min; 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec.

- b. Taqman Universal PCR master mix with SmartCycler
  - 2 × Taqman master mix: 12.5 µl
  - Forward primer: 2.5 µl (900 nM)/ Reverse primer: 2.5 µl (900 nM) / probe: 2.5 µl (250 nM)
  - H₂O: 2.5 µl / DNA template: 2.5 µl / Final volume: 25 µl
  - 95 °C for 10 min; 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec.

- c. IQ Supermix with IQ5 Real-Time PCR system
  - 2 × IQ Supermix master mix: 12.5 µl
  - Forward primer: 2.5 µl (900 nM)/ Reverse primer: 2.5 µl (900 nM) / Taqman probe: 2.5 µl (250 nM)
  - H₂O: 2.5 µl / DNA template: 2.5 µl / Final volume: 25 µl
  - 95 °C for 10 min; 40 cycles of 95 °C for 15 sec, 54 °C for 20 sec and 60 °C for 30 sec.

- d. IQ Supermix with SmartCycler
  - 2 × IQ Supermix master mix: 12.5 µl
  - Forward primer: 2.5 µl (900 nM) / Reverse primer: 2.5 µl (900 nM) / Taqman probe: 2.5 µl (250 nM)
  - H₂O: 2.5 µl / DNA template: 2.5 µl / Final volume: 25 µl
  - 95 °C for 10 min; 40 cycles of 95 °C for 15 sec, 52 °C for 20 sec and 60 °C for 30 sec.
Pre-collaborative Study

- **Inclusivity tests:** > 50 isolates of *E. sakazakii*
- **Exclusivity tests:** > 30 species of *non-E. sakazakii*
Pre-collaborative Study

- Spike samples with two different levels of inoculation: low and high. Duplicate samples were prepared for each method. Different isolates and infant formula were used.

- Each comparison:
  - high level of inoculation, no. samples per method:
    - 20×100 g samples, 3×10 g samples and 3×1 g samples
  - low level of inoculation: no. samples per method:
    - 3×300 g samples, 20×100 g samples and 3×33 g samples

(100 g samples were used for comparative validation; other samples were used for MPN analysis. 5 × negative controls were used for each comparison)

- Four comparisons performed:
  - Milk protein based formula / atypical *E. sakazakii* strain SK90
  - Soy protein based formula / atypical *E. sakazakii* strain ES626
  - Soy protein based formula / typical *E. sakazakii* strain ATCC 12868 and a competitive non-*E. sakazakii* strain E441.
  - Milk protein based formula / typical *E. sakazakii* strain ATCC 29544
Sample Preparation and Distribution

- Prepared by Silliker Inc.: Lyophilized strains prepared by proprietary methods and mixed with pre-screened infant formula samples, then held RT for 2 to 4 wks to stabilize.

- Distributed by FDA Moffett Center.

- Sample loads, negative controls were unknown to validation lab operators.
Statistical Analysis

- AOAC: the Microbiology Qualitative Method Statistics Guidelines for unpaired test portions
- Mantel-Haenszel Chi square formula for unpaired samples ($\chi^2$)
Results: Inclusivity Tests

- 100% positive by DFI, R&F and Real time PCR
- 2 isolates yield less than 50% by RAPID ID 32E.
- A combination of chromogenic agars and PCR would ensure no false negatives
Results: Exclusivity Tests

- False positive by DFI (4/42): ATCC 51713 (Buttiauzella noakiae), E440 (Enterobacter helveticus sp. nov), LMG 23732 (Enterobacter helveticus, sp. nov) and E908 (Enterobacter novel species).
- False positive by R&F (4/42): ATCC 51713 (Buttiauzella noakiae), ATCC 8090 (Citrobacter freundii), E440 (Enterobacter helveticus sp. nov), and E441(Enterobacter novel species)
- False positive by real time PCR (1/42): LMG 23730 (Enterobacter turicensis, sp. nov)
- False positive by API 20E (1/42) and RAPID ID 32E (0/42)
- A combination of all above would ensure no false positives.
# Results: Comparison Study

<table>
<thead>
<tr>
<th>Samples Level</th>
<th>MPN</th>
<th>No. Test Portions</th>
<th>Samples Positive</th>
<th>Chi Square</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>revised 24 h</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td><strong>Lot 1 Control</strong></td>
<td>NA</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>NA NA</td>
</tr>
<tr>
<td>High</td>
<td>3.045</td>
<td>20</td>
<td>19</td>
<td>17</td>
<td>1.08</td>
</tr>
<tr>
<td>Low</td>
<td>1.589</td>
<td>20</td>
<td>16</td>
<td>11</td>
<td>2.78</td>
</tr>
<tr>
<td><strong>Lot 2 Control</strong></td>
<td>NA</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>NA NA</td>
</tr>
<tr>
<td>High</td>
<td>0.779</td>
<td>20</td>
<td>11</td>
<td>6</td>
<td>2.49</td>
</tr>
<tr>
<td>Low</td>
<td>0.475</td>
<td>20</td>
<td>6</td>
<td>2</td>
<td>2.44</td>
</tr>
<tr>
<td><strong>Lot 3 Control</strong></td>
<td>NA</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>NA NA</td>
</tr>
<tr>
<td>High</td>
<td>0.793</td>
<td>20</td>
<td>9</td>
<td>3</td>
<td>4.18</td>
</tr>
<tr>
<td>Low</td>
<td>0.37</td>
<td>20</td>
<td>7</td>
<td>0</td>
<td>8.27</td>
</tr>
<tr>
<td><strong>Lot 4 Control</strong></td>
<td>NA</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>NA NA</td>
</tr>
<tr>
<td>High</td>
<td>2.727</td>
<td>20</td>
<td>19</td>
<td>1</td>
<td>31.59</td>
</tr>
<tr>
<td>Low</td>
<td>0.233</td>
<td>20</td>
<td>5</td>
<td>0</td>
<td>5.57</td>
</tr>
</tbody>
</table>
Another Message

Don’t always trust manufacturer's instructions or published journal articles

www.rf-labs.com
Conclusion

The revised FDA method is highly specific and sensitive and performs better than the reference FDA method for the detection of *E. sakazakii* in powered infant formula.
Acknowledgement

• Kwang-Young Song, Ph.D.
• Yi Chen, Ph.D.
• Eric Brown, Ph.D.
• Thomas Hammack