Development of Real-time PCR Screening Methods for the Detection of Bacterial Toxin DNA

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Overview

• Wadsworth Center Biodefense Laboratory
• Utility of rtPCR as a rapid screening method
• Staphylococcal enterotoxin rtPCR
• Clostridium botulinum rtPCR
• New and upcoming technology
### Biodefense Laboratory Services

#### Threats
- Powders, letters, “All Hazards”
- Biohazard Detection System

#### Foods
- Intentional/Unintentional

#### Animals
- Wild, domestic
- Outbreaks, investigations

#### Patients
- Naturally occurring
- Smallpox vs. Vaccination events
- Emerging Diseases (SARS, Flu)

#### Training
- Laboratorians
- First Responders

#### Assay Development
- Emerging diseases
- Novel technologies
- Optimized methods

#### Network Partnerships
- LRN
- FERN
- ERLN, ECLRN
- USPS, BDS
rtPCR Assay Development

- Follow standardized procedure in the laboratory, based on guidelines developed for clinical specimen testing
- Distinct development and validation phase
- Important to evaluate extraction and mastermix components of assay
- Assays were designed with multiplexing capability in mind
- Assays are validated as singleplex assays and evaluated to meet analytical criteria (sensitivity, specificity, etc.) and then diagnostic parameters are tested.
DNA Extraction Methods

**Manual (EpiCenter)**

- **Throughput**
  - 10 + Controls/batch

- **Time**
  - 2.5 hours/batch

- **Personnel**
  - BSL-2: 1 person
  - BSL-3: 2 people

**Automated***

- **Throughput**
  - 8-30 Controls/batch

- **Time**
  - 1.5 hrs hours/batch

- **Walk-away**

- **Personnel**
  - BSL-2: 1 person
  - BSL-3: 1 person

*Automated Extractions:

- EasyMag (BioMerieux) & MagNA Pure (Roche)
Detection limit at > 100 CFU/μL. Not sensitive enough.
Optimization of extraction procedure was necessary for both organism and food matrices.
# Singleplex rtPCR Assay Development

**S. aureus** Enterotoxin Type B

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Ave Ct value</th>
<th>% detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>undil</td>
<td>12.54</td>
<td>100%</td>
</tr>
<tr>
<td>10^-1</td>
<td>15.77</td>
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<tr>
<td>10^-2</td>
<td>19.59</td>
<td>100%</td>
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<tr>
<td>10^-3</td>
<td>23.15</td>
<td>100%</td>
</tr>
<tr>
<td>10^-4</td>
<td>26.42</td>
<td>100%</td>
</tr>
<tr>
<td>10^-5</td>
<td>29.59</td>
<td>100%</td>
</tr>
<tr>
<td>10^-6</td>
<td>32.96</td>
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<tr>
<td>10^-7</td>
<td>36.54</td>
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<tr>
<td>10^-8</td>
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<td>10^-9</td>
<td>38.52</td>
<td>19%</td>
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<tr>
<td>10^-10</td>
<td>37.87</td>
<td>6%</td>
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</table>

**LOD**

10^-7 dilution = ~ 1 CFU/rxn
Identification of SEB toxin DNA in food-borne outbreak

Identification of cluster of prison inmates sickened after consuming egg salad
Multiplex Real-Time PCR

- Rapid turn-around times
- Multiple toxins types screened
- Minimal volumes needed
- Streamlines testing with more labor-intensive techniques
- Highly sensitive, specific and reliable
- Must be carefully designed and developed
Detecting Staph Enterotoxin DNA

- rtPCR multiplex reaction: Toxin Types A, B and E

<table>
<thead>
<tr>
<th>Final Reagent Concentrations</th>
<th>SaEA</th>
<th>SaEB</th>
<th>SaEE</th>
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<tr>
<td>MgCl(_2)</td>
<td>5.0 mM</td>
<td>5.0 mM</td>
<td>5.0 mM</td>
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<tr>
<td>Forward Primer</td>
<td>150 nM</td>
<td>75 nM</td>
<td>50 nM</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>75 nM</td>
<td>75 nM</td>
<td>150 nM</td>
</tr>
<tr>
<td>Dual Labeled Probe</td>
<td>50 nM</td>
<td>50 nM</td>
<td>150 nM</td>
</tr>
<tr>
<td>Roche Master Mix Buffer</td>
<td>1x</td>
<td>1x</td>
<td>1x</td>
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</table>
Detection of SaEB in Food using multiplex rtPCR (A,B, and E)

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Extraction Method</th>
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<tbody>
<tr>
<td></td>
<td>Epicentre</td>
</tr>
<tr>
<td>Undiluted</td>
<td>14.14</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>17.30</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>20.29</td>
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<tr>
<td>$10^{-3}$</td>
<td>23.71</td>
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<tr>
<td>$10^{-4}$</td>
<td>27.21</td>
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<tr>
<td>$10^{-5}$</td>
<td>31.18</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>Undetected</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>Undetected</td>
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</tbody>
</table>
# Detection of SaEB in Food

<table>
<thead>
<tr>
<th>Extraction Method</th>
<th>Food Matrix</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Egg Salad</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epicentre</td>
<td>7.5 cfu/ rxn</td>
<td>1.25 cfu/ rxn</td>
<td>10 cfu/ rxn</td>
<td></td>
</tr>
<tr>
<td>MagNA Pure</td>
<td>7.5 cfu/ rxn</td>
<td>12.5 cfu/ rxn</td>
<td>10 cfu/ rxn</td>
<td></td>
</tr>
<tr>
<td>EasyMAG</td>
<td>675 cfu/ rxn</td>
<td>11.3 cfu/ rxn</td>
<td>900 cfu/ rxn</td>
<td></td>
</tr>
</tbody>
</table>
Staph. Enterotoxin Food Poisoning Cluster
(March 2009)

- 2 Adults: 1 male & 1 female, mean age 47 years
- Specimens received:
  - 2 Primary stool samples: 1 from each patient
  - 1 implicated food source from residence (noodle mixture)
- *Staphylococcus* culture & toxin typing requested
  - *Multiplex rtPCR assays*
Multiplex rtPCR ABI 7500 SaEA, B & E

Delta Rn vs Cycle

SaEA

Food Sample Wash

Positive Controls

Food Sample Homogenate

Cycle Number
Multiplex rtPCR ABI 7500 SaEC, D & TSST

Delta Rn vs Cycle

- TSST
- Food Sample Wash
- Positive Controls
- Food Sample Homogenate

Cycle Number

Delta Rn
- 1.0e+06
- 1.0e+05
- 1.0e+04
- 1.0e+03
- 1.0e+02
- 1.0e+01

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45
Results:

• Direct food & patient sample testing rtPCR positive for SaEA & SaTSST

• Subsequent isolates also rtPCR positive SaEA & SaTSST

• SaEA results confirmed by TECRA & Ochterlony testing
Clinical vs. Environmental Samples

• Clinical samples:
  – Stool, sputum, blood, serum, tissues, urine...

• Environmental samples:
  – Animals, food, powders, water, dirt...

• Common Problems:
  – Inhibition
  – High background of normal flora
  – May have limited sample volume
Botulism Neurotoxins

• Challenges in detection
  - Food
  - Environmental
  - Multiple toxin types

• Challenges in identification classically
  - Very small sample volumes
  - Gold standard: Mouse Bioassay
    • Difficult, time consuming, requires large samples or isolated organism
  - Turn around times are critical for public health
Method Optimization
Bot Toxins

• Automated extraction assists in removal of inhibitors
  • Avoids gel formation in certain samples, filter clogging with some extraction kits

• Heat denaturation
  - Initial step at 95°C for 30min
    • Protein denaturation, toxin inactivation, lysis begins

• Spore Filter as a Safety Enhancement
  - Final step: spin extract through spore filter
  - Ensures all viable organism are removed
Evolution of *C. botulinum* PCR

- **2000**: Conventional PCR
- **2002**: Singleplex rtPCR
- **2005**: Multiplex rtPCR Assays
Multiplex rtPCR (Triplex and Duplex) Assays
Botulinum Toxin Types A, B, E, and F + Baratii F
<table>
<thead>
<tr>
<th>Date</th>
<th>Sample</th>
<th>Sample Type</th>
<th>PCR Result</th>
<th>Bioassay Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>09-17-03</td>
<td>03-5016</td>
<td>Infant Stool</td>
<td>B</td>
<td>Insufficient material</td>
</tr>
<tr>
<td>09-23-03</td>
<td>03-5028</td>
<td>Infant Stool</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>02-09-04</td>
<td>04-1490</td>
<td>Stool</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>02-12-04</td>
<td>04-1491</td>
<td>Serum</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>07-14-04</td>
<td>04-7755</td>
<td>Infant Stool</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>01-11-05</td>
<td>05-239</td>
<td>Gastric Juices</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>03-03-06</td>
<td>06-2064</td>
<td>Infant Stool</td>
<td>B</td>
<td>Insufficient material</td>
</tr>
<tr>
<td>03-06-06</td>
<td>06-2105</td>
<td>Infant Stool</td>
<td>B</td>
<td>Insufficient material</td>
</tr>
<tr>
<td>05-01-07</td>
<td>07-3738</td>
<td>Infant Stool</td>
<td>B</td>
<td>Insufficient material</td>
</tr>
<tr>
<td>05-02-07</td>
<td>07-3970</td>
<td>Infant Stool</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>05-14-07</td>
<td>07-4010</td>
<td>Adult Stool</td>
<td>C. baratii F</td>
<td>F</td>
</tr>
<tr>
<td>05-25-07</td>
<td>07-4354</td>
<td>Adult Stool</td>
<td>C. baratii F</td>
<td>F</td>
</tr>
<tr>
<td>06-06-07</td>
<td>07-4605</td>
<td>Infant Stool</td>
<td>B</td>
<td>B</td>
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<tr>
<td>07-24-07</td>
<td>07-5854</td>
<td>Infant Stool</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>10-02-07</td>
<td>07-7376</td>
<td>Infant Stool</td>
<td>B</td>
<td>Insufficient material</td>
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<tr>
<td>10-12-07</td>
<td>07-7660</td>
<td>Infant Rectal Swab</td>
<td>B</td>
<td>Insufficient material</td>
</tr>
</tbody>
</table>
Recent Infant Botulism Case Involving FERN Laboratories

- NYSDOH notified on 3/2/09 of suspect case
- Infant was seen by Dr on 2/25/09-diagnosed with ear infection and given abx
- Baby taken to hospital on 2/28/09
- Baby transferred to another hospital after suspicion of botulism after ruling out other CNS causes on 3/2/09
- Specimens sent to WC on 3/2/09
- Received at WC on 3/3/09
- rtPCR testing completed 3/3/09 PM with a result of C. bot Type A DNA positive
- Investigation as to determine of botulism-baby never fed food but did receive a herbal remedy
contains catnip leaf, fenugreek seed, peppermint leaf, nettles, Elixir, Limm Balm, Chamomile, Ginger, spring water and a bit of grain alcohol.

- Product made in a Amish/Mennonite community
- Hold placed on product distribution
- WC received the remedy 3/4/09 and tested negative
- Baby recovering-out of ICU- 3/6/09

Mouse bioassay results: Positive for patient, negative for herbal remedy

- 3/7/09-Montana DPHHS collected samples; also herbal extracts in CA collected and tested by FDA-WA
- 4/14/09- Testing completed by FDA- all samples were negative
Application of New Technology: Microarrays
Akonni/Wadsworth Collaboration

Preparation
TruTip™ (< 5 min)

PCR / Detection
TruArray® (< 2.5 hr)
  Biothreat Assay
  Viral encephalitis Assay
  TB

Reporting
TruDx™1000 Imager (< 2 min)
Application of the Current Technology

Microarray of Biodefense Targets

21 Array features in replicates of 4, DNA probes 25-30mers

<table>
<thead>
<tr>
<th>PCR amplicon (BP)</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td><em>Ricinus communis</em></td>
</tr>
<tr>
<td>126</td>
<td><em>Orthopoxvirus</em></td>
</tr>
<tr>
<td>130</td>
<td><em>Bacillus anthracis</em></td>
</tr>
<tr>
<td>150</td>
<td><em>Clostridium bot. AB</em></td>
</tr>
<tr>
<td>150</td>
<td><em>Clostridium bot. F</em></td>
</tr>
<tr>
<td>125</td>
<td><em>Francisella tularensis</em></td>
</tr>
<tr>
<td>112</td>
<td><em>Brucella abortus</em></td>
</tr>
<tr>
<td>110</td>
<td><em>Staphylococcus aureus tox. B</em></td>
</tr>
<tr>
<td>120</td>
<td><em>Burkholderia mallei</em></td>
</tr>
<tr>
<td>114</td>
<td><em>Clostridium perfringens</em></td>
</tr>
<tr>
<td>123</td>
<td><em>Rickettsia prowazekii</em></td>
</tr>
</tbody>
</table>
Single Target Assay Microarray
Dual Priming 11-Plex Assay with control singleplex (SP)

Target Agent
*Brucella abortus* Spot # 15
On-chip PCR

- PCR, hybridization wash and imaging in a single chamber.
- No “open” amplicon in the workspace.
- DNA and RNA detection.
  - Demonstrated on B. anthracis, Y. pestis, M. tuberculosis, S. pyogenes, Influenza A, Influenza B.
Conclusions

• rtPCR extremely valuable as a screening tool
• Cost-effective method to test multiple samples and organisms isolated from these samples
• Decreases number of animals needed for mouse bioassay confirmation
• Currently performing studies in different food matrices for validation of the *C. botulinum* assay
• New technology will also decrease time and labor for unknown causes of foodborne illness
Acknowledgements

- Staph Enterotoxin Extractions/PCR
  - Mrs. Amy Chiefari

- Botulism Toxins Extractions/PCR
  - Mr. Stephen Davis

- Botulism Mouse Bioassay
  - Mr. George Hannett
  - Mr. Stephen Davis

- Dr. Nick Cirino