The Molecular Detection Toolbox: Applications and Implications on Current and Future National Monitoring Efforts

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LT2 Rule: Cryptosporidium Analytical Method Improvements and Update on Source Water Monitoring
7 December 2011
Overview

I. Protozoan molecular detection toolbox
   • Molecular genotyping: then and now
   • Application of molecular methods for detecting *Cryptosporidium*
   • Strategies to integrate molecular assays with USEPA Method 1623

II. Advantages, limitations, and future considerations
DNA → PCR → Genes → Genomes
Can we use these breakthroughs for compliance monitoring?

1953

1983/1988: PCR/commercialization

1988:

1992: Real-time PCR

1995:

Microarray

2001:

C. parvum genome

2001:

Pyrosequencing ("Next-gen" sequencing)

2004:

C. hominis genome

2007:

C. muris genome

2007 Human microbiome

1998:

2007 Human microbiome

Current status:
- Personal Genome Project /Knome
- Personal genome service ("know your DNA" $100)
- >10,000 Genomes submitted to NCBI
- >300 Metagenome projects (>70% Environmental)
End-point vs. real-time PCR

End-point PCR

- Semi-quantitative (densitometry)
- Can amplify longer sequences
- Very specific
- Sequencing compatible

Real-time PCR

- Quantitative/standard curve
- Fluorescent probe
- Short PCR product (amplicon)
- Very specific

Foodnet.org
Molecular diagnostic tools (“genotyping”) are widely used

- **Food and waterborne disease outbreak investigations**
  - Drinking water (*C. hominis*)
  - Sprouts (*E. coli O104:H4*)
  - Raspberries (*C. cayetanensis*)
  - Waterparks (*C. hominis*)

- **Clinical diagnostics**
  - HIV
  - Breast cancer (BRCA 1/2)
  - Tuberculosis
  - MRSA

- **Ecology**
  - Zebra/Quagga mussels
  - Other invasive species
Molecular detection of Cryptosporidium

- PCR-based detection tools are increasing
- PCR for detection and genotyping
  - Real-time quantitative PCR for detection
  - Microarrays for multi-pathogen detection
- Identifying sources of contamination
  - Adult cattle vs. calves
  - Zoonoses vs. anthroponoses
Molecular detection of Cryptosporidium

- Impact of drinking water regulations on cryptosporidiosis outbreaks
- Specific C. parvum subtypes correlates with Method 1623 performances (Using PCR for Q/C issues)
- First Cryptosporidium qPCR kits available in ~1998
Molecular-based assays, does it fit into USEPA Method 1623?

I. Collection/Filtration

II. Secondary Concentration

III. Detection

Microscopic enumeration

Disinfection Profiling and Benchmarking

After completing the initial round of source water monitoring any system that plans on making a significant change to their disinfection practices must:

- Create disinfection profiles for *Giardia lamblia* and viruses;
- Calculate a disinfection benchmark; and,
- Consult with the state prior to making a significant change in disinfection practice.

<table>
<thead>
<tr>
<th>Cryptosporidium Concentration (oocysts/L)</th>
<th>Bin Classification</th>
<th>Additional Cryptosporidium Treatment Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 0.075</td>
<td>Bin 1†</td>
<td>No additional treatment required</td>
</tr>
<tr>
<td>0.075 to &lt; 1.0</td>
<td>Bin 2</td>
<td>1 log, 1.5 log, 1 log</td>
</tr>
<tr>
<td>1.0 to &lt; 3.0</td>
<td>Bin 3</td>
<td>2 log, 2.5 log, 2 log</td>
</tr>
<tr>
<td>≥ 3.0</td>
<td>Bin 4</td>
<td>2.5 log, 3 log, 2.5 log</td>
</tr>
</tbody>
</table>
Molecular-based Cryptosporidium monitoring?

Approaches to integrate Molecular typing with “Method 1623”

- 1- Off-the-bead typing and quantitation
  - Real-time PCR
  - Genus or species specific
- 2- Off-the-slide genotyping
  - Also quantitative (microscopic)
  - Identifies genus/species/genotype

Sample Collection

Concentration

1

2
Detection of *Cryptosporidium* spp. oocysts using Taqman-based qPCR

<table>
<thead>
<tr>
<th>Primer sets</th>
<th><em>Cryptosporidium</em> spp.</th>
<th><em>C. hominis</em></th>
<th><em>C. parvum</em></th>
<th>CRULib13</th>
<th>CRULib13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>JVA CRU18S Pan18S Ch001 Ch003 JVAG1 Ch</td>
<td>Cp001 Cp003 JVAG2 Cp</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Protozoa</strong></td>
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<tr>
<td><em>C. parvum</em></td>
<td>+ + + +</td>
<td>- - - -</td>
<td>+ + + + + +</td>
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<tr>
<td><em>C. hominis</em></td>
<td>+ + + +</td>
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<td>- - - - - -</td>
<td>- - - -</td>
<td>- - - -</td>
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<tr>
<td><em>C. meleagris</em></td>
<td>+ + + +</td>
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<td>- - - - - -</td>
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<tr>
<td><em>C. felis</em></td>
<td>+ + + +</td>
<td>- - - - - -</td>
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<tr>
<td><em>C. canis</em></td>
<td>- + + +</td>
<td>- - - - - -</td>
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<td>- - - -</td>
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<tr>
<td><em>C. muris</em></td>
<td>- + + +</td>
<td>- - - - - -</td>
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<tr>
<td><em>G. muris</em></td>
<td>- - - -</td>
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<td><em>G. duodenalis</em></td>
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<td><em>T. gondii</em></td>
<td>- + + +</td>
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<tr>
<td><strong>Bacteria</strong></td>
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<td><em>B. thuringiensis</em></td>
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<td><em>B. cereus</em></td>
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<td><em>E. coli</em></td>
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<td>- - - -</td>
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<tr>
<td><em>S. flexneri</em></td>
<td>- - - -</td>
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<td>- - - -</td>
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<tr>
<td><strong>Fungi</strong></td>
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<tr>
<td><em>E. hellem</em></td>
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<tr>
<td><em>E. intestinalis</em></td>
<td>- - - -</td>
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<td>- - - -</td>
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<tr>
<td><em>E. cuniculi</em></td>
<td>- - - -</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
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<td>- - - -</td>
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<tr>
<td><strong>Helminth</strong></td>
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<tr>
<td><em>S. mansoni</em></td>
<td>- ND ND</td>
<td>- - - - - -</td>
<td>ND - - - -</td>
<td>- - - -</td>
<td>ND - - -</td>
</tr>
</tbody>
</table>

ND, Not done
Detection of spiked *Cryptosporidium* spp. oocysts in environmental samples

### C. *parvum* specific qPCR

<table>
<thead>
<tr>
<th>Oocysts</th>
<th>Primer/Probe set</th>
<th>Cryptosporidium spp. specific</th>
<th>C. <em>parvum</em> specific</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>JVA CRU18S</td>
<td>Cp003 JVAG2 CRULib13 Cp</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>37.41 ± 1.03 (9/9) 37.66 ± 1.47 (7/9)</td>
<td>38.01 ± 0.99 (3/9) 37.02 ± 0.72 (9/9) 37.96 ± 1.16 (4/9)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>38.45 ± 0.82 (7/9) 37.14 ± 0.68 (7/9)</td>
<td>* 37.34 ± 1.08 (4/9) 38.27 ± 0.23 (2/9)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>38.98 ± 0.59 (4/9) 37.26 ± 1.14 (9/9)</td>
<td>*</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>38.14 ± 0.48 (2/9) 36.61 ± 1.10 (9/9)</td>
<td>* 38.02 (1/9) *</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>* 36.94 ± 1.06 (9/9)</td>
<td>* *</td>
</tr>
</tbody>
</table>

* Dinoflagellate cross-reactive

### C. *hominis* specific qPCR

<table>
<thead>
<tr>
<th>Oocysts</th>
<th>Primer/Probe set</th>
<th>Cryptosporidium spp. specific</th>
<th>C. <em>hominis</em> specific</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>JVA CRU18S</td>
<td>Ch003 JVAG1 CRULib13 Ch</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>39.58 (1/9) 31.57 ± 1.09 (9/9)</td>
<td>38.29 ± 0.72 (5/9) 38.91 ± 0.29 (2/9) 37.51 ± 0.42 (4/9)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>32.56 ± 0.22 (3/9) 31.81 ± 0.74 (9/9)</td>
<td>38.88 ± 0.64 (4/9) *</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>* 32.91 ± 0.93 (9/9)</td>
<td>37.71 ± 0.17 (3/9) 38.41 (1/9) *</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>* 32.99 ± 0.91 (9/9)</td>
<td>39.24 (1/9) *</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>* 33.41 ± 0.87 (9/9)</td>
<td>* *</td>
</tr>
</tbody>
</table>

Summary and limitations

- **C. hominis/parvum specific qPCR assay**
  - Specific to *C. hominis/parvum* species
  - Limit of detection 1-10 oocysts
  - Poor resolution at low oocyst concentration
    - Cannot distinguish between 1, 2, or 5 oocysts

- **Does not identify exotic/emerging pathogenic genotypes**
  - e.g., skunk, horse or *C. cuniculus*
  - No *Cryptosporidium* genus specific qPCR (to date)

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How useful is it for Method 1623?
Off-the-slide molecular detection of *Cryptosporidium* species

Sample Collection

Concentration

Molecular typing

Off-the-slide scraping DNA extraction

Enumeration

✓ Genotyping (PCR-Sequencing)

Quantitation
Off-the-slide genotyping reliability, sensitivity, and genotypes detected

- **C. andersoni**
- **C. ryanae**
- **C. baileyi**
- **C. bovis**
- **C. parvum**
- **C. hominis**
- **C. spp. SW 1-5**

- **C. ubiquitum**
- **C. xiaoi**
- **fox genotype**
- **Genotype W1/12**
- **Muskrat I/II**
- **C. muris**

- Average *Cryptosporidium* oocyst levels detected:
  - 0.09-0.26 oocysts/L (Bin 1-2)

- Does not identify the source(s) of contamination

Reucker, et.al. 2007
Nichols, et.al. 2010

Nichols, et.al. 2010

Ware and Villegas. 2011. In preparation
A technique that builds on Method 1622/1623, which can identify *Cryptosporidium* species based on unique sequences in their genetic code.

Low cost capital and reagents for conducting molecular genotyping assays.
Off-the-slide molecular detection of Cryptosporidium species

Human pathogenic
- C. hominis
- C. parvum
- C. meleagridis
- C. ubiquitum

Animal associated
- C. andersoni
- C. serpentis
- C. muris
- C. baileyi
- NTC

Overall detection rates (multi-lab)

Giovanni, G. personal comm.
• Provides additional information on species/genotypes detected via Method 1623
  • Nucleic acid vs. oocyst?

  • “The slide genotyping method has not been approved by the USEPA… And does not currently have regulatory significance.”
1. How do we assess *Cryptosporidium* spp. diversity
   - Molecular based approaches

2. What are the total levels of *Cryptosporidium*
   - Method 1623, or qPCR? (resolution dependent)

3. What are the total levels of pathogenic *Cryptosporidium*
   - Molecular based approaches

4. Are the *Cryptosporidium* oocysts viable/infectious
   - Cell culture, vital dyes, or mouse bioassay

5. What are the levels of viable/infectious *Cryptosporidium*
   - Cell culture or vital dye + qPCR

6. Other questions…
   - Custom built using the “*Cryptosporidium* detection toolbox”
Factors to consider for a Cryptosporidium molecular method

- Molecular vs. Microscopy
  - Performance comparison, capital equipment, lab capacity, and cost
  - Nucleic acid vs. oocyst detection

- Sensitivity, specificity, and precision
  - 1-4 oocysts/L, 5-10 oocysts/L
  - Target gene(s) (copy numbers and multiple loci)
  - Internal controls
  - Genus vs. species specific

- Confounding factors:
  - Indigenous naked DNA/PCR inhibitors
  - qPCR platform
  - Reagent cross reactivity

- Standardization and validation of protocol
  - Commercialization of reagents/equipment
  - Quality assurance/control guidelines

- Repository for genetic information: environmental and clinical isolates

USEPA approval
Adoption of the method
The evolution of molecular detection technologies

1. Molecular-based detection of waterborne pathogens continues to evolve
   • Already at the point where the entire genome can be sequenced in 1 week

2. Provides the means to better understand the prevalence, source(s), and genotypes of microbial pathogens in water

Is it only only a matter of time?..
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- Cristin Brescia
- Scott Keely
- David Erisman

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- Jiim Ferretti

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**NCEA**
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**CDC**

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- Wenli Yang
- Vitaliano Cama
- Theresa Dearen

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- Leah Villegas

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- Abu Sayed
- Reena Mackwan
Questions?

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