EPA
United States
Environmental Protection
Agency

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

Eric N. Villegas, Ph.D.

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

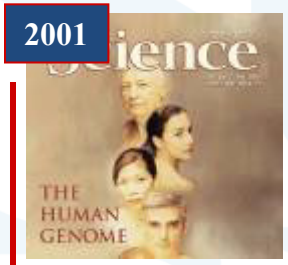
DNA → PCR → Genes → Genomes

Can we use these breakthroughs for compliance monitoring?

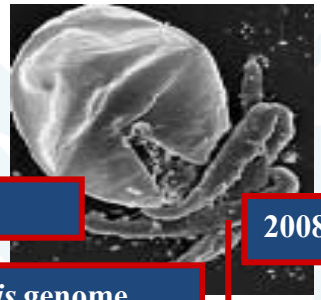
1953

1998

2001



2001: *C. parvum* genome



2008: *C. muris* genome

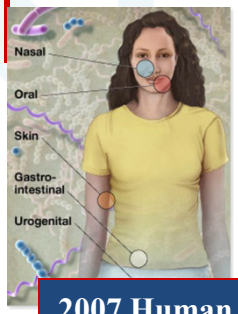
2004: *C. hominis* genome

1983/1988: PCR/commercialization

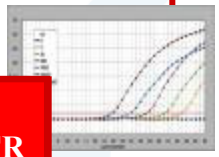
1995: Microarray

2007

2005: Pyrosequencing ("Next-gen" sequencing)



2007 Human microbiome



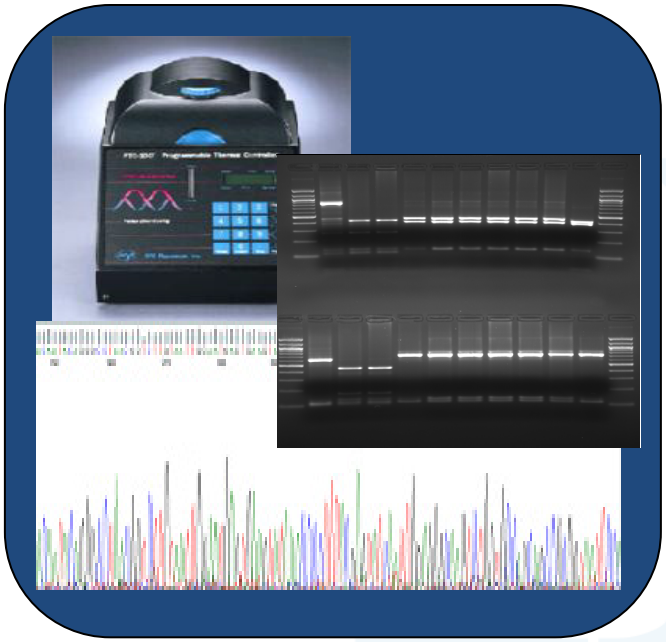
1992: Real-time PCR

Current status:

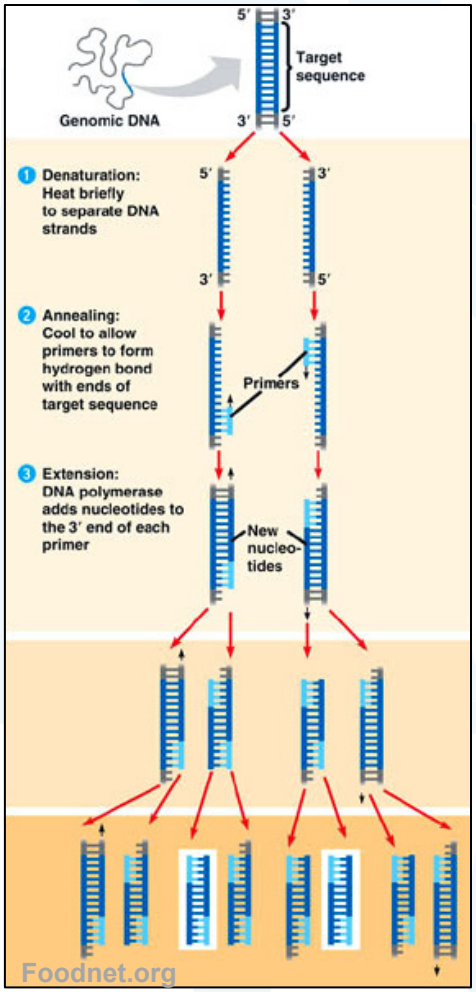
- Personal Genome Project /Knome
- Personal genome service ("know your DNA" $100)
- >10,000 Genomes submitted to NCBI
- >300 Metagenome projects (>70% Environmental)

End-point vs. real-time PCR

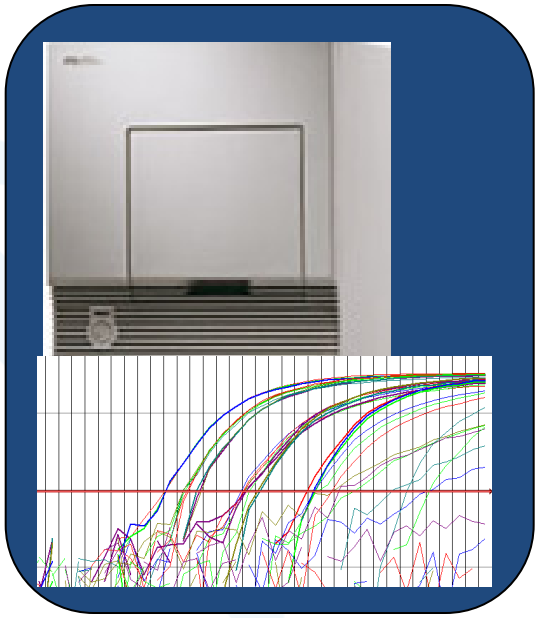
End-point PCR



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



Real-time PCR

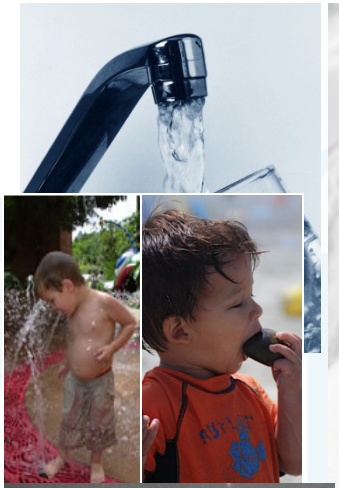


- Quantitative/standard curve
- Fluorescent probe
- Short PCR product (amplicon)
- Very specific

Molecular diagnostic tools ("genotyping") are widely used

- **Food and waterborne disease outbreak investigations**

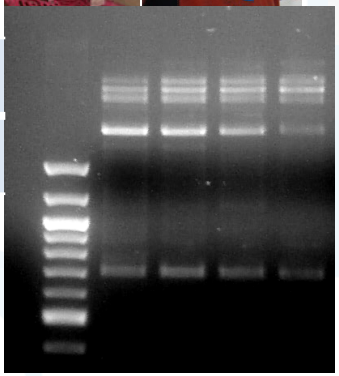
• Drinking water (*C. hominis*)	• Sprouts (*E. coli O104:H4*)
• Raspberries (*C. cayetanensis*)	• Waterparks (*C. hominis*)

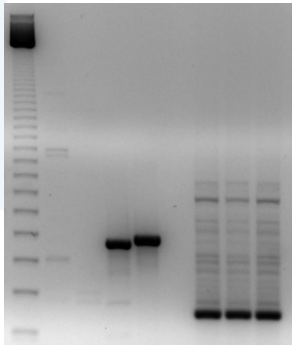


http://goowedding.blogspot.com/2011/05/wedding-in-washington-dc-raspberry.html0

- **Clinical diagnostics**

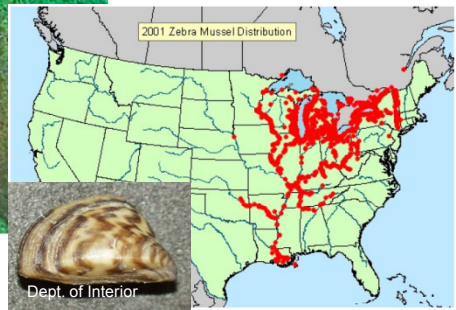
• HIV	• Breast cancer (BRCA 1/2)
• Tuberculosis	• MRSA





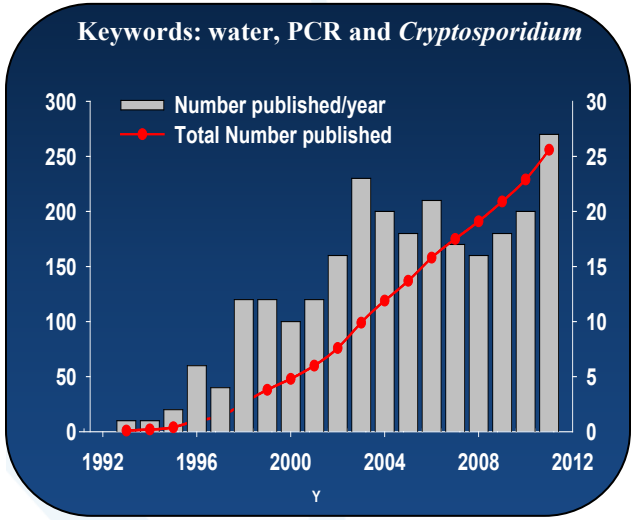
- **Ecology**

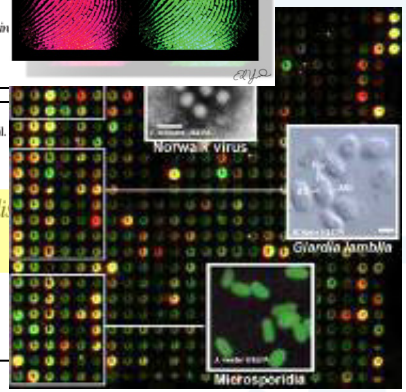
• Zebra/Quagga mussels	• Other invasive species



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





Applied and Environmental Microbiology, Apr. 2002, p. 1817–1826
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.4.1817–1826.2002
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Genotyping *Cryptosporidium parvum* with an *hsp70* Single-Nu
Polymorphism Microarray

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Detection and Genotyping of *Entamoeba histolytica*, *Entamoeba di*
Giardia lamblia, and *Cryptosporidium parvum* by
Oligonucleotide Microarray

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FEMS MICROBIOLOGY Letters
www.fems-microbiology.org

Development of a TaqMan quantitative PCR assay specific for
Cryptosporidium parvum

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www.elsevier.com/locate/jmicmeth

gnetic separation–real-time PCR method for
f *Cryptosporidium parvum* in water samples

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of *Cryptosporidium* species from human faeces

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Journal of Clinical Microbiology, July 2002, p. 2335–2338
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.7.2335–2338.2002

Detection and Differentiation of *Cryptosporidium* Parasites That Are
Pathogenic for Humans by Real-Time PCR

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ptosporidium
in Environmental Water Samples and Sewage

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Detection and differentiation of *Cryptosporidium hominis* and *Cryptosporidium parvum* by dual TaqMan assays

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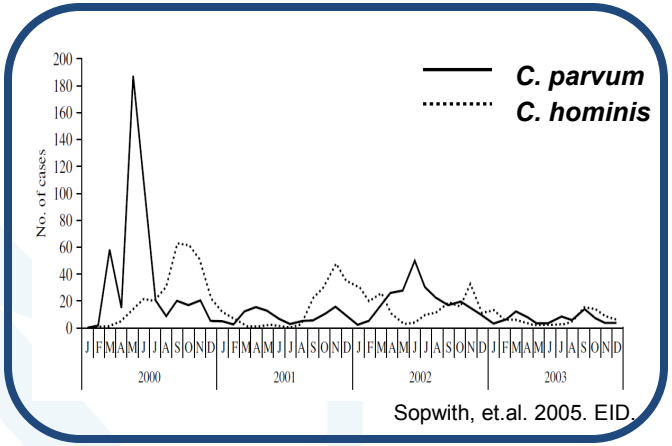
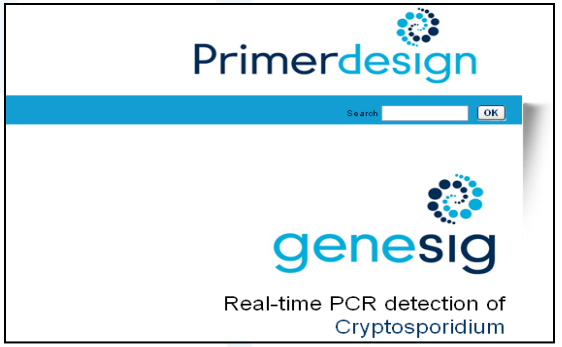
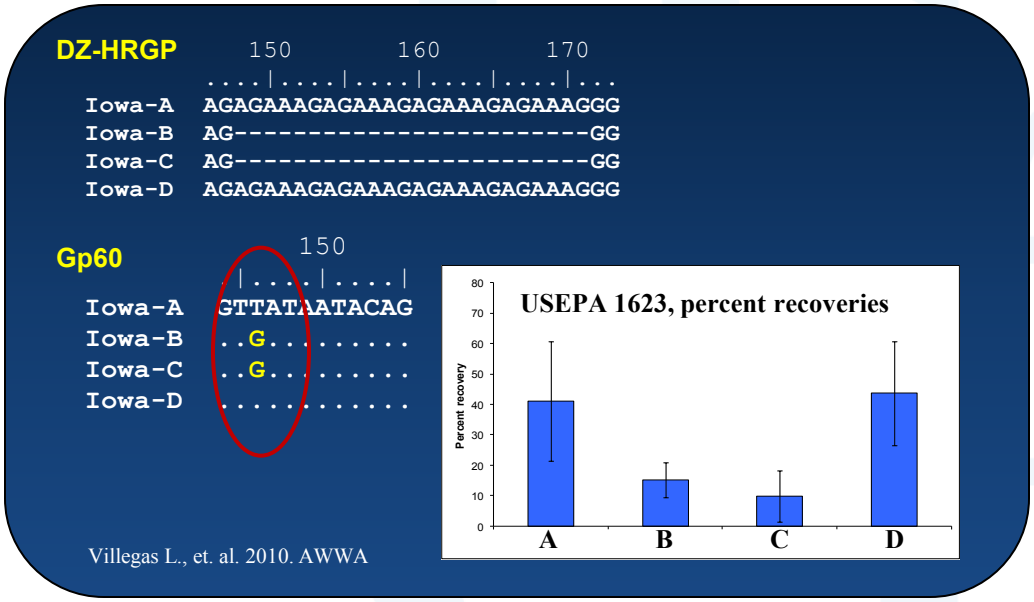
Molecular detection of *Cryptosporidium*

- **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**

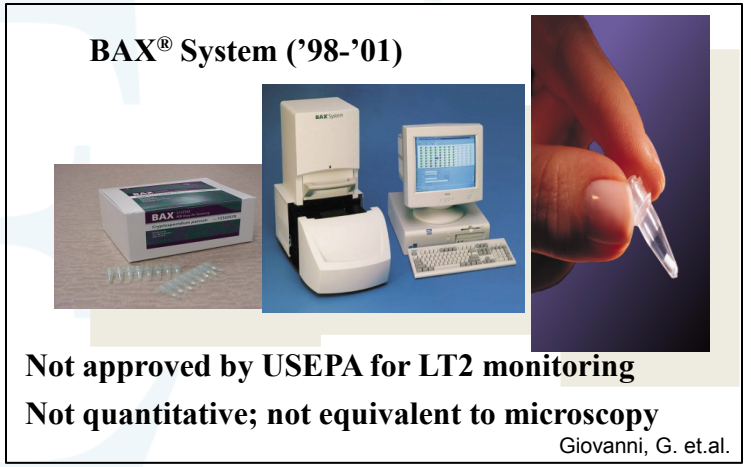


Sopwith, et.al. 2005. EID.





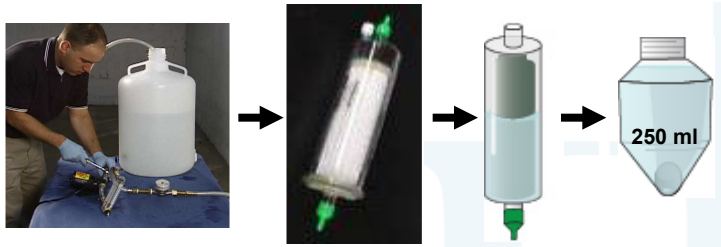
Villegas L., et. al. 2010. AWWA



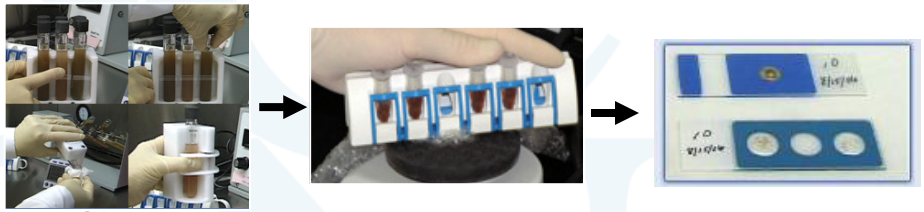
Giovanni, G. et.al.

Molecular-based assays, does it fit into USEPA Method 1623?

I. Collection/Filtration

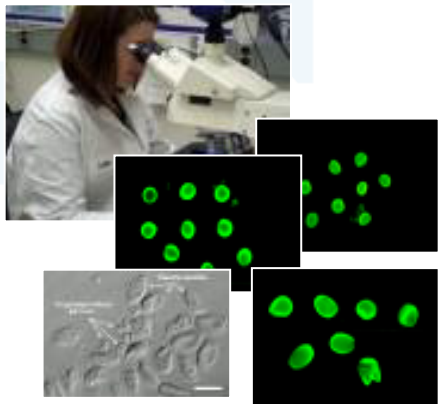


II. Secondary Concentration



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.

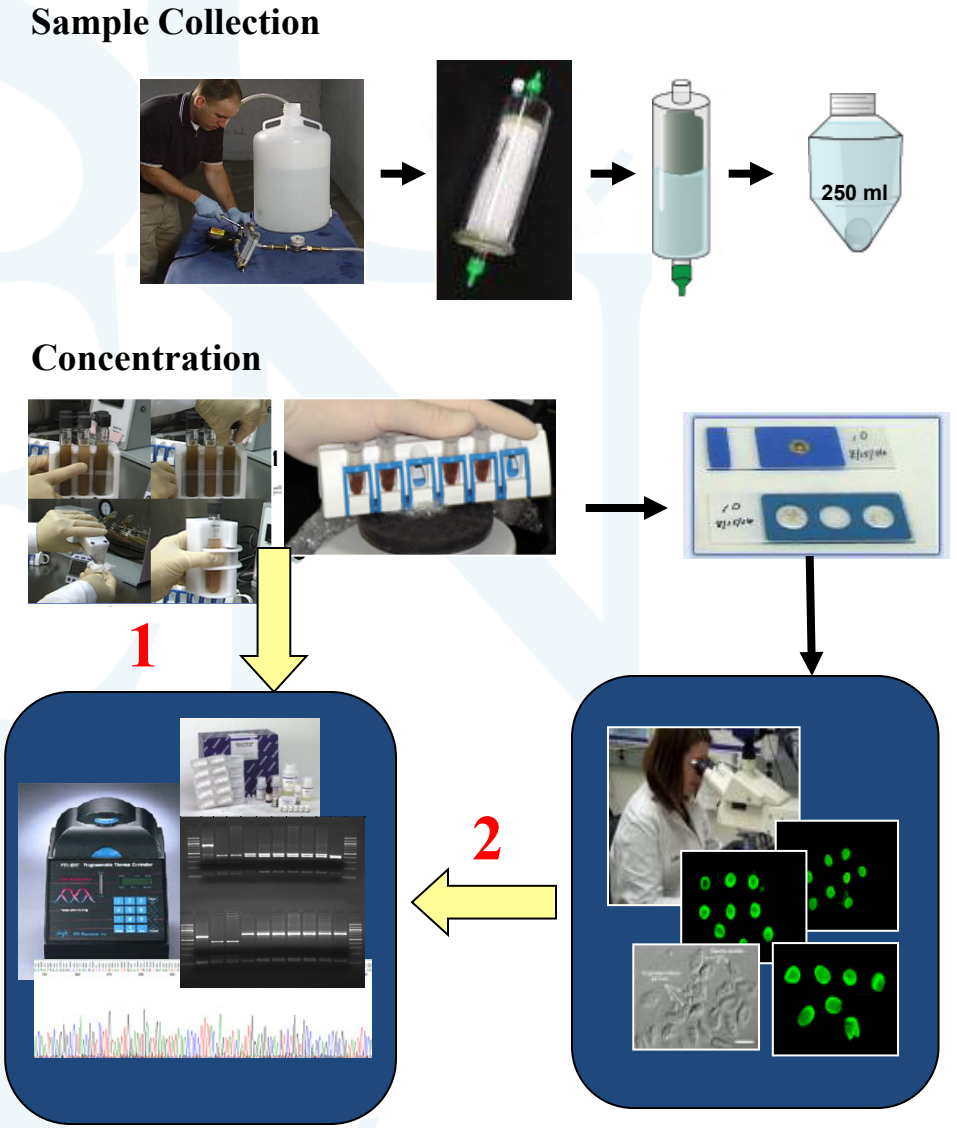
Bin Classification For Filtered Systems

Cryptosporidium Concentration (oocysts/L)	Bin Classification	Additional *Cryptosporidium* Treatment Required			Alternative Filtration
		Conventional Filtration	Direct Filtration	Slow Sand or Diatomaceous Earth Filtration	
< 0.075	Bin 1††	No additional treatment required			
0.075 to < 1.0	Bin 2	1 log	1.5 log	1 log	(1)
1.0 to < 3.0	Bin 3	2 log	2.5 log	2 log	(2)
≥ 3.0	Bin 4	2.5 log	3 log	2.5 log	(3)

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



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

Species	Primer sets: *Cryptosporidium spp.* JVA	CRU18S	Pan18S	*C. hominis* Ch001	Ch003	JVAG1	CRULib13 Ch	*C. parvum* Cp001	Cp003	JVAG2	CRULib13 Cp
Protozoa											
C. parvum	+	+	+	-	-	-	-	+	+	+	+
C. hominis	+	+	+	+	+	+	+	-	-	-	-
C. meleagridis	+	+	+	+	-	-	-	-	-	-	-
C. felis	+	+	+	-	-	-	-	-	-	-	-
C.canis	-	+	+	-	-	-	-	-	-	-	-
C. muris	-	+	+	-	-	-	-	-	-	-	-
G. muris	-	-	-	-	-	-	-	-	-	-	-
G. duodenalis	-	-	-	-	-	-	-	-	-	-	-
T. gondii	-	+	+	-	-	-	-	-	-	-	-
Bacteria											
B. thuringiensis	-	-	-	-	-	-	-	-	-	-	-
B. cereus	-	-	-	-	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-	-	-	-
S. flexneri	-	-	-	-	-	-	-	-	-	-	-
Fungi											
E. hellem	-	-	-	-	-	-	-	-	-	-	-
E. intestinalis	-	-	-	-	-	-	-	-	-	-	-
E. cuniculi	-	-	-	-	-	-	-	-	-	-	-
Helminth											
S. mansoni	-	ND	-	-	-	-	ND	-	-	-	ND

ND, Not done

Staggs and Villegas. 2011. In preparation.

Detection of spiked *Cryptosporidium* spp. oocysts in environmental samples

C. parvum specific qPCR

	Primer/Probe set				
	Cryptosporidium spp. specific		*C. parvum* specific		
Oocysts	JVA	CRU18S	Cp003	JVAG2	CRULib13 Cp
10	37.41 ± 1.03 (9/9)	37.66 ± 1.47 (7/9)	38.01 ± 0.99 (3/9)	37.02 ± 0.72 (9/9)	37.96 ± 1.16 (4/9)
5	38.45 ± 0.82 (7/9)	37.14 ± 0.68 (7/9)	*	37.34 ± 1.08 (4/9)	38.27 ± 0.23 (2/9)
2	38.98 ± 0.59 (4/9)	37.26 ± 1.14 (9/9)	*	*	38.04 (1/9)
1	38.14 ± 0.48 (2/9)	36.61 ± 1.10 (9/9)	*	38.02 (1/9)	*
0	*	36.94 ± 1.06 (9/9)	*	*	*

Dinoflagellate cross-reactive

C. hominis specific qPCR

	Primer/Probe set				
	Cryptosporidium spp. specific		*C. hominis* specific		
Oocysts	JVA	CRU18S	Ch003	JVAG1	CRULib13 Ch
10	39.58 (1/9)	31.57 ± 1.09 (9/9)	38.29 ± 0.72 (5/9)	38.91 ± 0.29 (2/9)	37.51 ± 0.42 (4/9)
5	32.56 ± 0.22 (3/9)	31.81 ± 0.74 (9/9)	38.88 ± 0.64 (4/9)	*	38.36 (1/9)
2	*	32.91 ± 0.93 (9/9)	37.71 ± 0.17 (3/9)	38.41 (1/9)	*
1	*	32.99 ± 0.91 (9/9)	39.24 (1/9)	*	*
0	*	33.41 ± 0.87 (9/9)	*	*	*

Staggs and Villegas. 2011. In preparation.

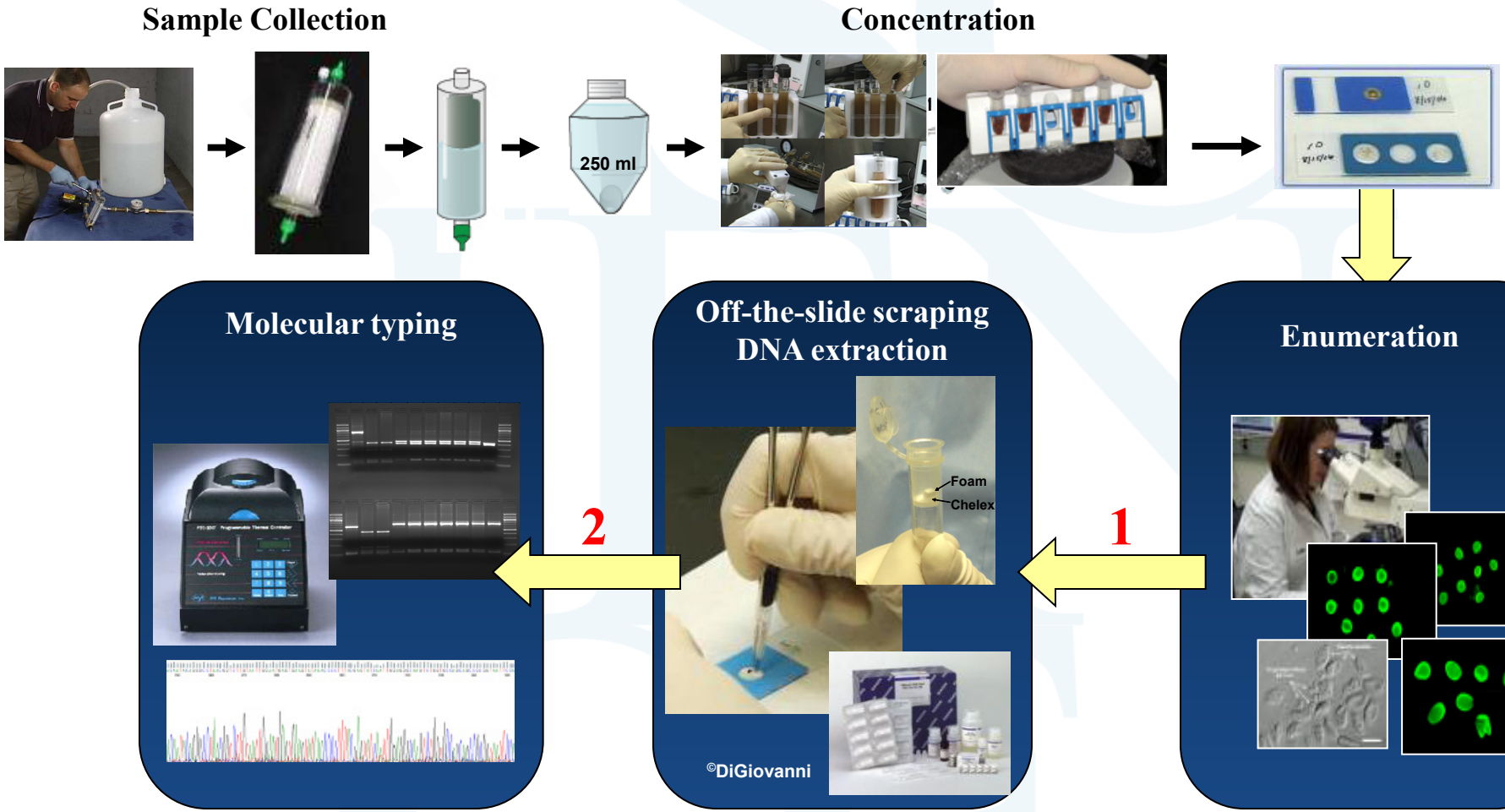
Summary and limitations

- ***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)**

How useful is it for Method 1623?

Off-the-slide molecular detection of *Cryptosporidium* species

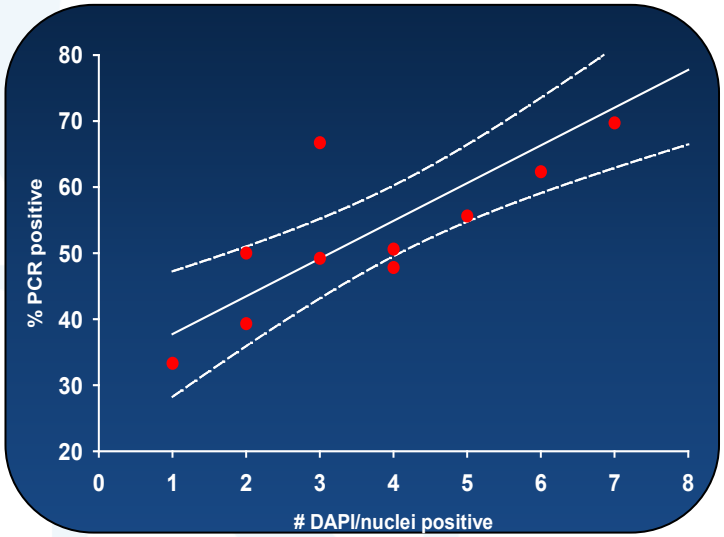


Off-the-slide genotyping reliability, sensitivity, and genotypes detected

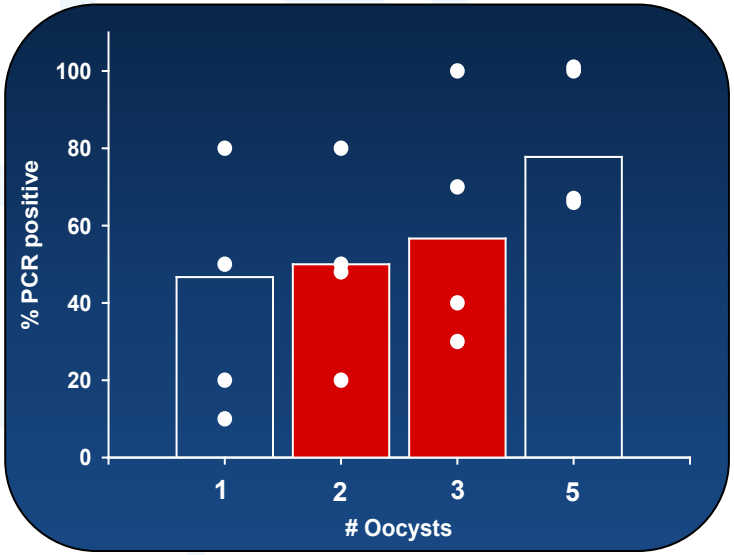
- *C. andersoni*
- *C. ryanae*
- *C. baileyi*
- *C. bovis*
- *C. parvum*
- *C. hominis*
- *C. spp.* SW 1-5
- *C. ubiquitum*
- *C. xiaoi*
- fox genotype
- Genotype W1/12
- Muskrat I/II
- *C. muris*

- 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

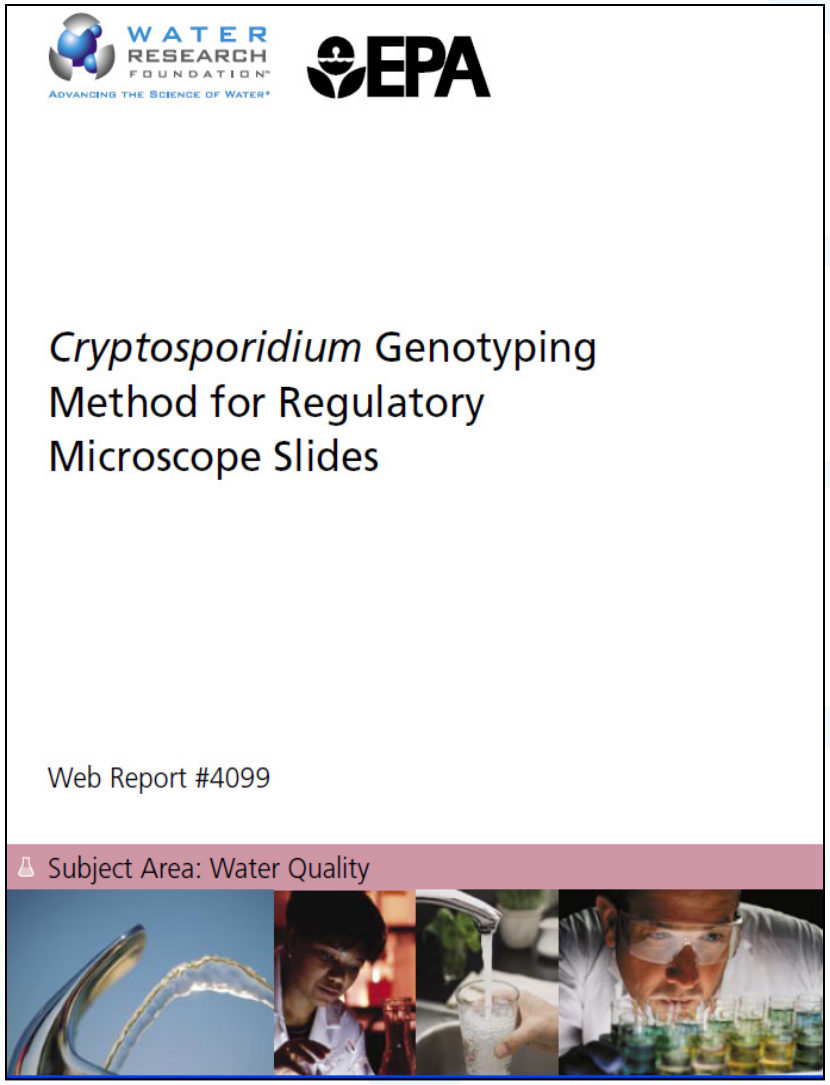


Nichols, et.al. 2010



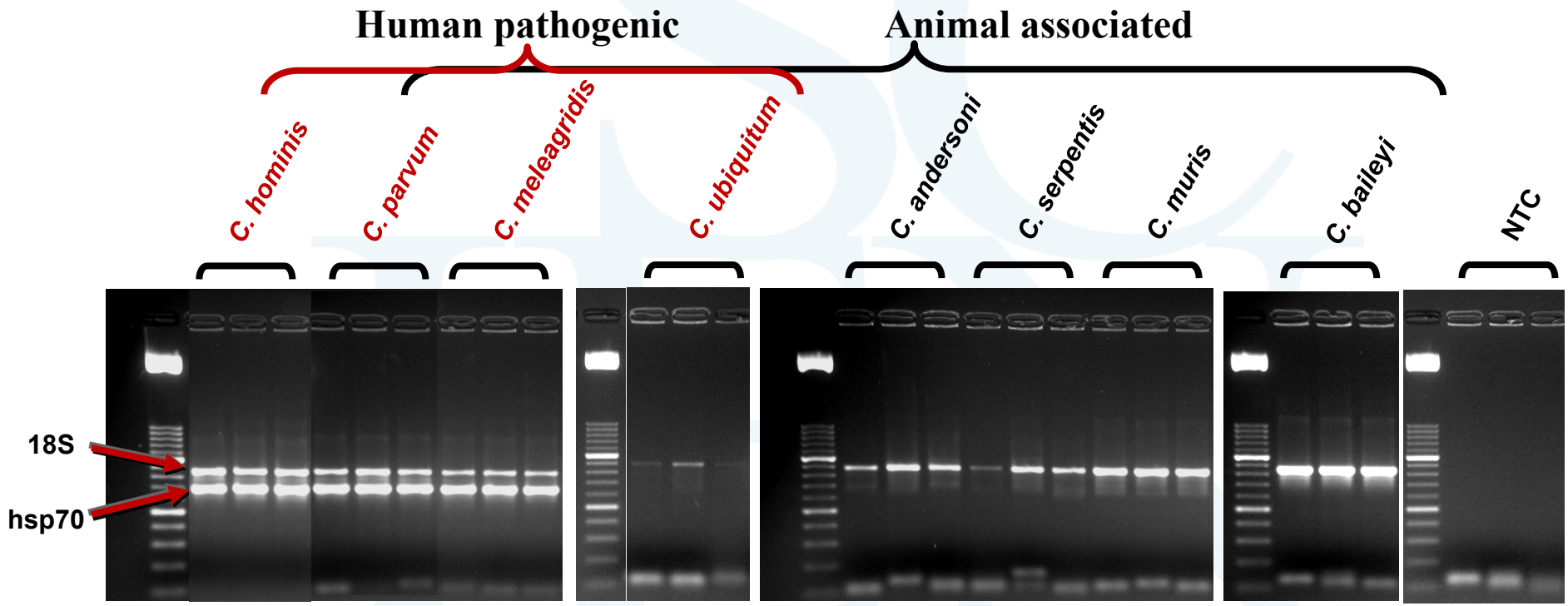
Ware and Villegas. 2011. In preparation

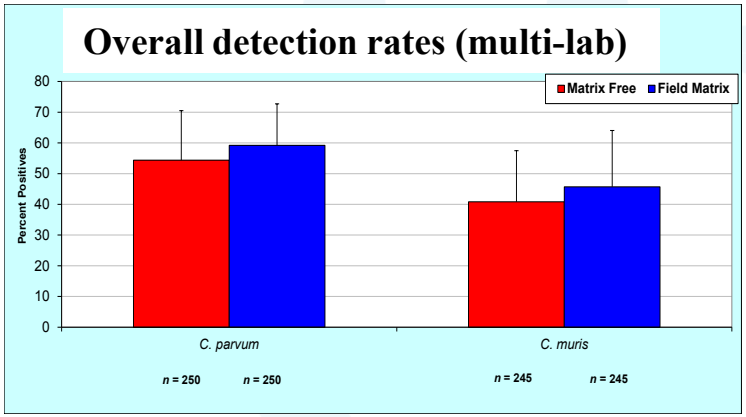
Water Research Foundation: Off-the-slide genotyping method (4099)



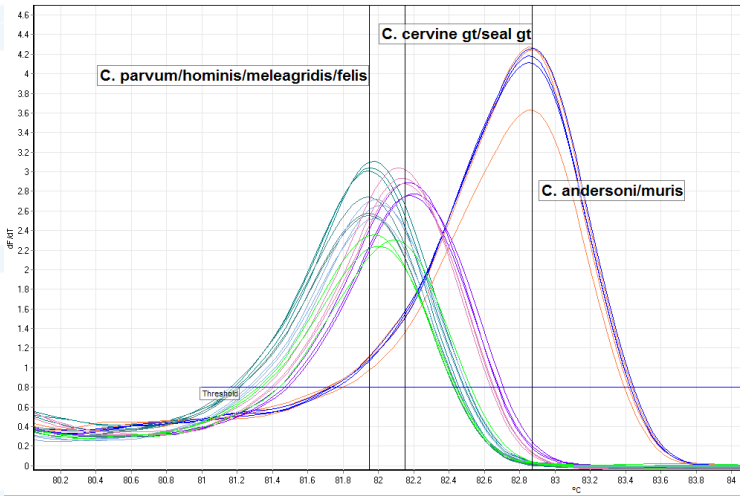
- "A technique that builds on Method 1622/1623, which can identify *Cryptosporidium* species based on unique sequences in their genetic code."
- Low cost capital and reagents for conducting molecular genotyping assays

Off-the-slide molecular detection of *Cryptosporidium* species

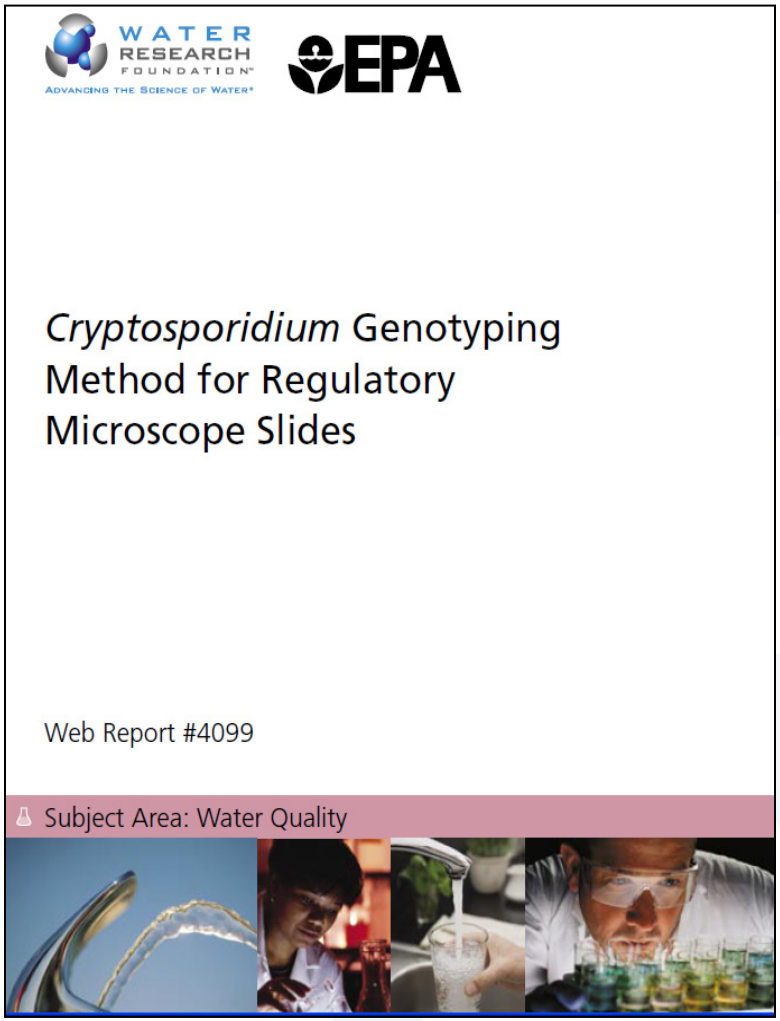




Giovanni, G. personal comm.



Summary



- Provides <u>additional information</u> 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."

Cryptosporidium monitoring efforts: Must be question (NOT assay) driven

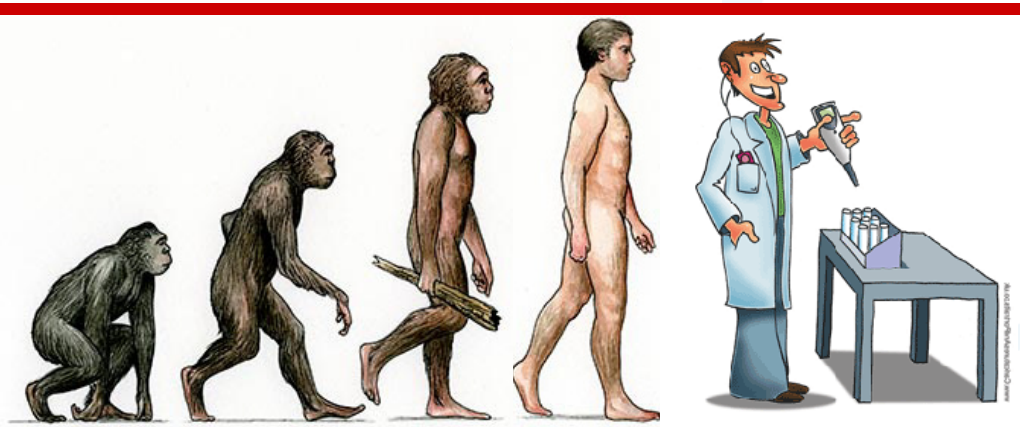
1. How do we assess *Cryptosporidium* spp. diversity
   - Molecular based approaches
2. What are the total levels of *Cryptosporidium*
   - Method 1623, or qPCR? (resolution dependent)
3. What are the total levels of pathogenic *Cryptosporidium*
   - Molecular based approaches
4. Are the *Cryptosporidium* 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



http://www.molecularstation.com/molecular-biology-images/509-pcr-pictures/71-thermocycler-old-pcr-machine.html

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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