United States Environmental Protection Agency

Surveillance Systems for Waterborne Protozoa: Beyond Method 1623

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April 24, 2009

Office of Research and Development
National Exposure Research Laboratory | Microbiological and Chemical Exposure Assessment Research Division | Biohazard Assessment Research Branch

Topics to be Discussed

1. Brief introduction to waterborne *Cryptosporidium* and *Giardia*
   - Historical perspective on detecting *Cryptosporidium* and *Giardia*
   - Current detection methodologies
2. US EPA's waterborne protozoan research program
   - Building a "Protozoan Detection Toolbox"
3. Perspectives on the future of the "Protozoan Detection Toolbox"
   - Future directions
   - Factors to consider for developing a pathogen specific detection method

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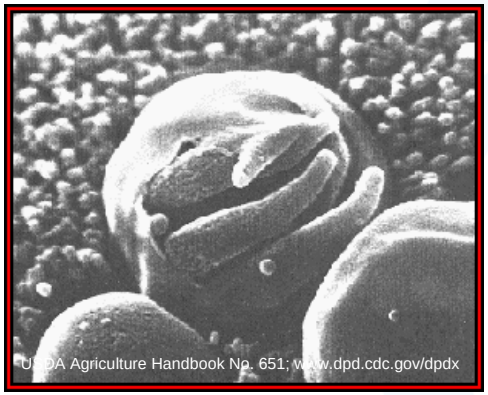
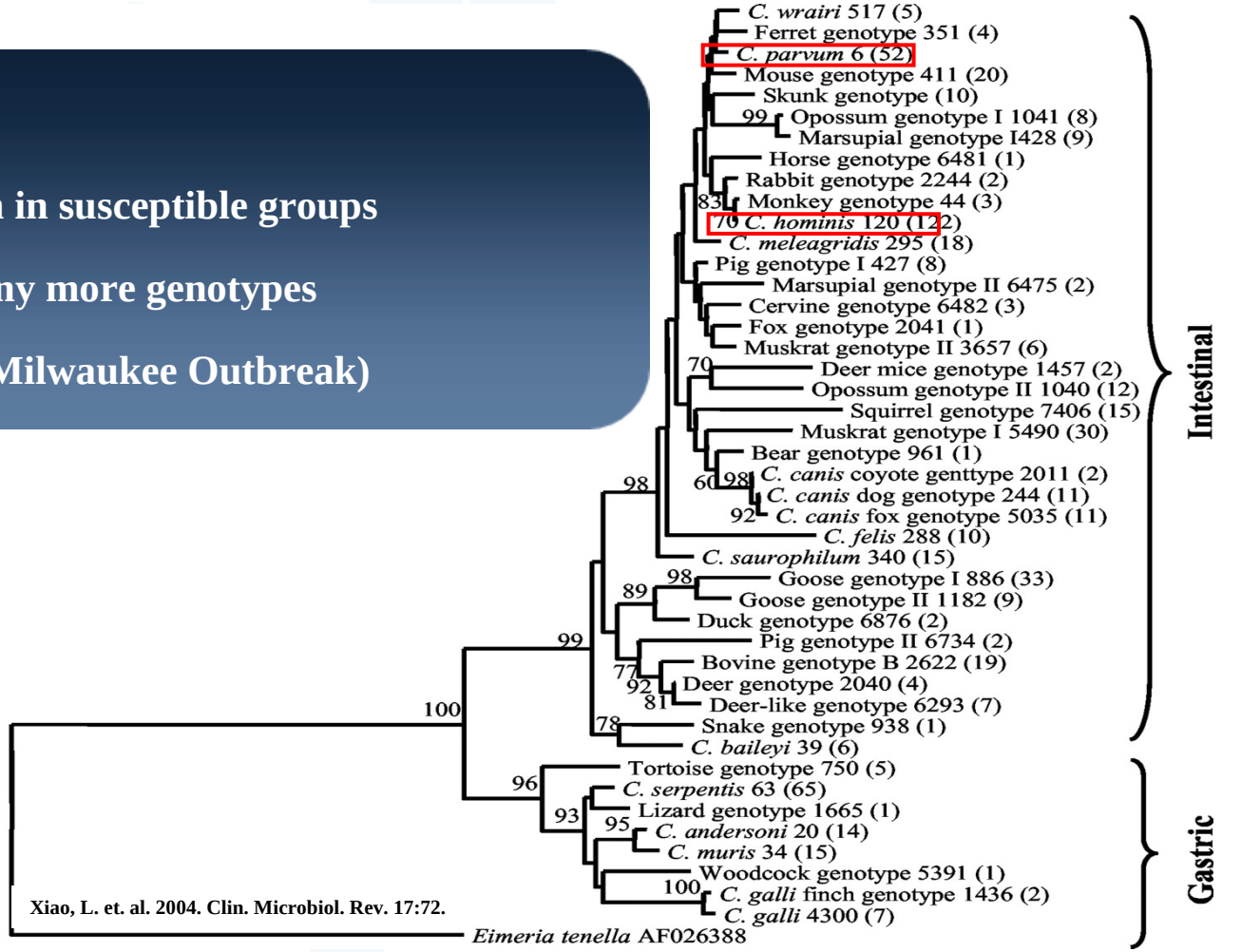
Cryptosporidium species

- Enteric protozoan parasite
- Chronic diarrhea and death in susceptible groups
- At least 20 species, with many more genotypes
- Waterborne transmission (Milwaukee Outbreak)





Cryptosporidium Species Infecting Humans and Selected Animals

Host	Major Species	Minor Species
Humans	*C. hominis and C. parvum* (90% of all infections)	*C. meleagridis, C. felis, C. canis, C. suis, cervine genotype*
Cat	*C. felis*	
Cattle	*C. parvum, C. bovis, C. andersoni, deer-like genotype*	*C. suis*
Chickens	*C. baileyi*	*C. meleagridis*
Deer	*C. parvum, deer genotype*	
Dog	*C. canis*	
Turkey	*C. meleagridis, C. baileyi*	
Pig	*C. suis*	*Pig genotype II*
Sheep	*Cervine genotype 1-3, bovine genotypes*	

Modified from Fayer and Xiao. 2008.

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US EPA Drinking Water Regulations for *Cryptosporidium* and *Giardia*

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Surface Water Treatment Rule (1990)

The SWTR, which became effective on December 31, 1990, requires all systems using surface water, or ground water under the direct influence of surface water, to disinfect. It also requires all such systems to filter their water unless they can demonstrate that they have an effective watershed protection program and can meet other EPA-specified requirements. The SWTR also specifies that systems using surface water must treat water to remove/inactivate at least 99.9% (3 $\log_{10}$) of the *Giardia lamblia* cysts (a protozoan) and at least 99.99% (4 $\log_{10}$) of the viruses. The SWTR does not require a system to monitor its source water or drinking water for these pathogens.

Interim Enhanced Surface Water Treatment Rule (1998)

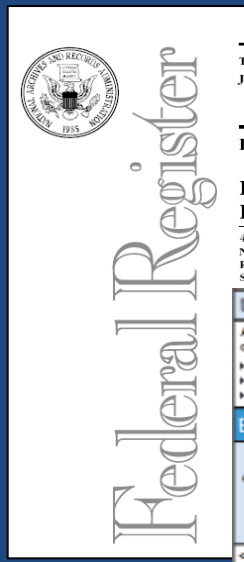
SUMMARY: In this document, EPA is finalizing the Interim Enhanced Surface Water Treatment Rule (IESWTR). The purposes of the IESWTR are to: Improve control of microbial pathogens, including specifically the protozoan *Cryptosporidium*, in drinking water; and address risk trade-offs with disinfection byproducts. Key provisions established

Long Term 1 Enhanced Surface Water Treatment Rule (2002)

SUMMARY: In this document, EPA is finalizing the Long Term 1 Enhanced Surface Water Treatment Rule (LT1ESWTR). The purposes of the LT1ESWTR are to improve control of microbial pathogens, specifically the protozoan *Cryptosporidium*, in drinking water and address risk trade-offs with disinfection byproducts. The rule will

(DBPR). The LT1ESWTR applies to public water systems that use surface water or ground water under the direct influence of surface water and serve fewer than 10,000 persons. The LT1ESWTR builds upon the framework established for systems serving a population of 10,000 or more in the Interim Enhanced Surface Water Treatment Rule (IESWTR). This rule

Long Term 2 Enhanced Surface Water Treatment Rule (2006)



Federal Register

Thursday,
January 5, 2006

Part II

Environmental Protection Agency

40 CFR Parts 9, 141, and 142
National Primary Drinking Water Regulations: Long Term 2 Enhanced Surface Water Treatment Rule; Final Rule

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:

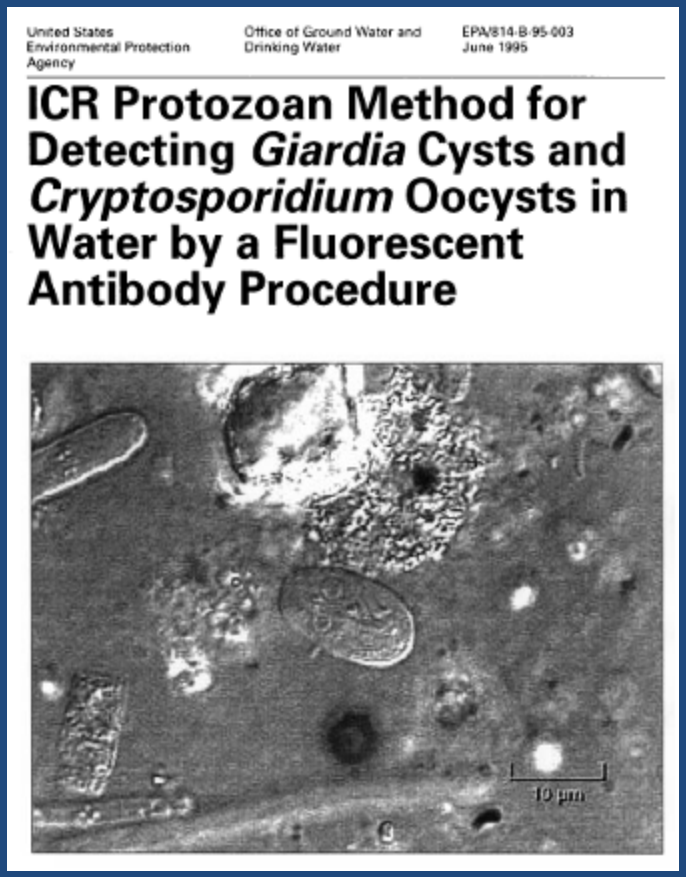
- Create disinfection profiles for *Giardia lamblia* and viruses;
- Calculate a disinfection benchmark; and,
- Consult with the state prior to making a significant change in disinfection practice.

Bin Classification For Filtered Systems

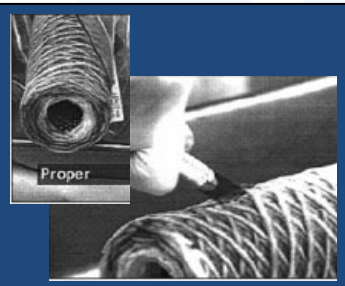
Cryptosporidium Concentration (oocysts/L)	Bin Classification	Additional *Cryptosporidium* Treatment Required			Alternative Filtration
		Conventional Filtration	Direct Filtration	Slow Sand or Diatomaceous Earth Filtration	
< 0.075	Bin 1	No additional treatment required	No additional treatment required	No additional treatment required	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)

Detection of *Cryptosporidium* and *Giardia*: "Then"

United States Environmental Protection Agency

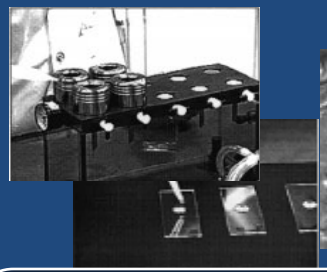

United States Environmental Protection Agency | Office of Ground Water and Drinking Water | EPA/814-B-95-003 June 1995

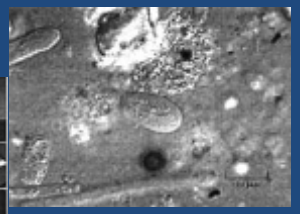
ICR Protozoan Method for Detecting *Giardia* Cysts and *Cryptosporidium* Oocysts in Water by a Fluorescent Antibody Procedure



Sample Collection Elution

Concentration Floataion



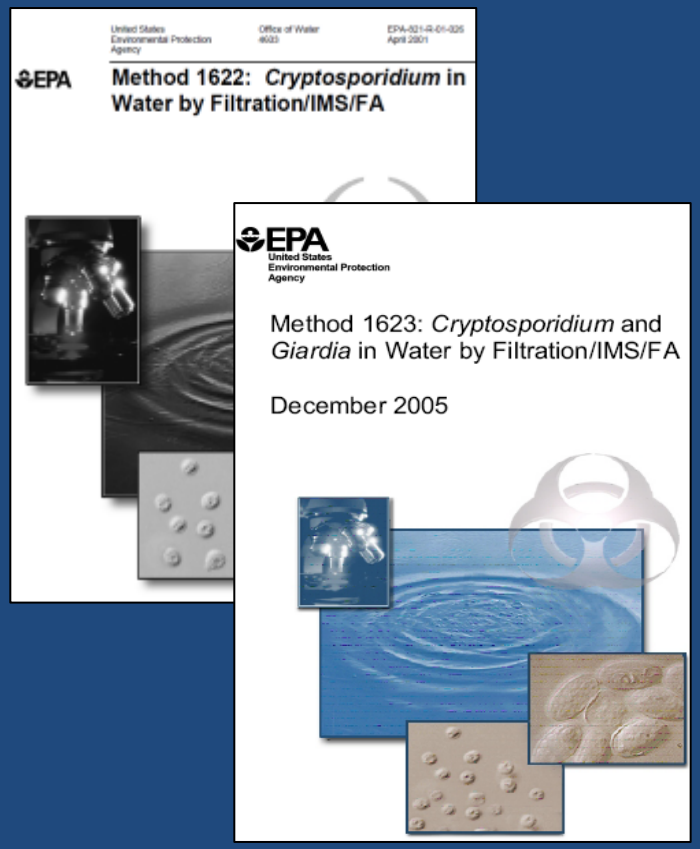


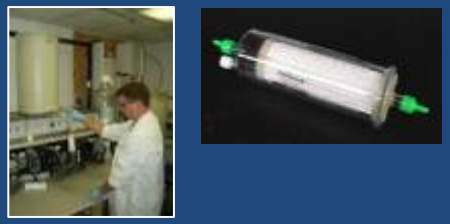
Immunofluorescence Detection

Limitations:

- **Poor recovery, reproducibility and sensitivity**
- **High limits of detection**

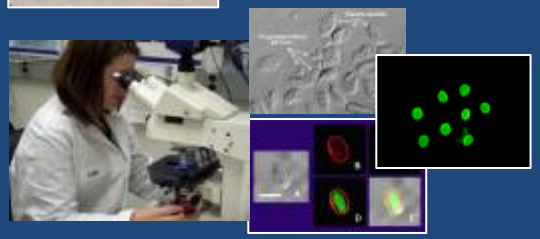
Method 1622/1623: Detection of *Cryptosporidium* and *Giardia* "Now"





Sample Collection
Elution

Immunomagnetic
Separation

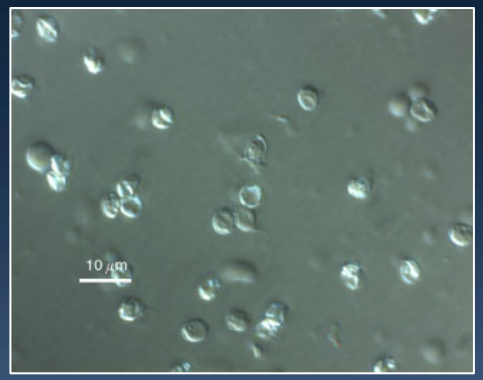


Immunofluorescence
Detection

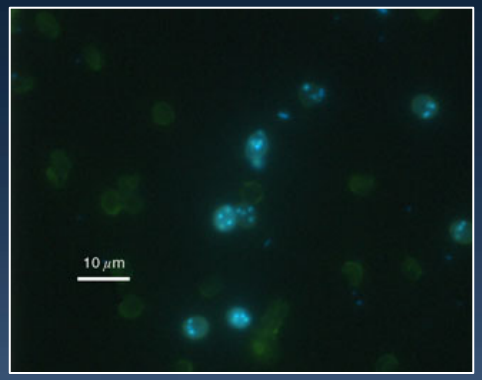
Method 1622/1623: Detection of *Cryptosporidium* Oocysts

A microscopic based detection method

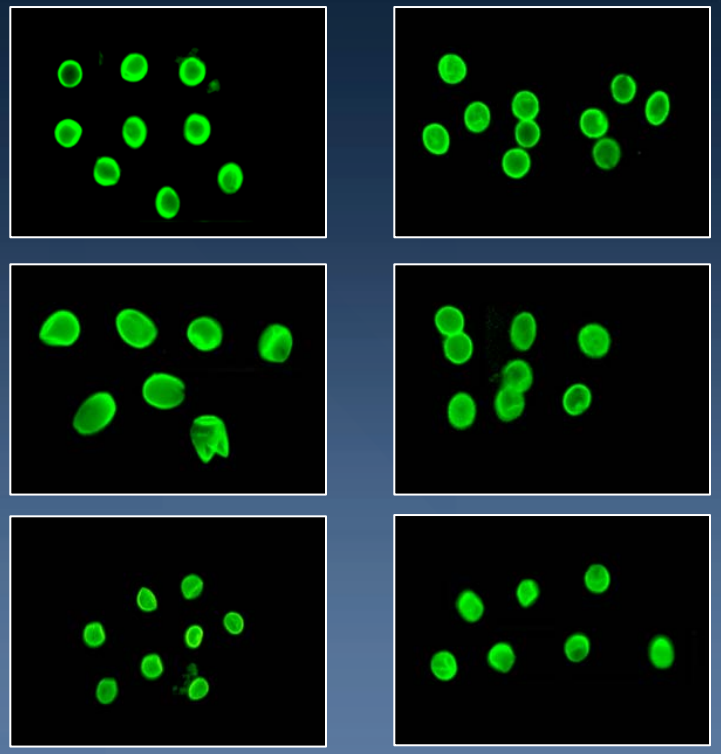
DIC



DNA staining



Cryptosporidium staining



Limitations:

- Does not differentiate human infectious vs. animal forms
- No live vs. dead discrimination

Images from Ware, EPA and Xiao, CDC

Challenges for the 21st Century "Is there a Silver Bullet?!"

Protozoan Detection Systems:

1. Fast and user friendly
2. Sensitive and quantitative
3. Species/genotype specific
4. Live vs. dead

What are the Questions?

1. What are the total levels of *Cryptosporidium/Giardia* in the watershed?
2. What are the total levels of pathogenic *Cryptosporidium/Giardia* in the watershed?
3. How complex is the *Cryptosporidium/Giardia* species diversity in the watershed?
4. Are the *Cryptosporidium/Giardia* oocysts in the watershed viable/infectious?
5. Other questions…

Is there Room for Improvements?

Method Now

- Improve filtration and concentration
- New technology to capture multiple pathogens
- New IMS reagents
  - antibodies
  - magnetic beads
- Improve recovery
  - Dissociation
- New reagents
  - Antibodies
- Cell Culture
- Molecular-based assays

Method of the Future

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Advances in the Detection of *Cryptosporidium* Oocysts and *Giardia* cysts in Water

Towards Developing a Complete "Protozoan Detection Toolbox"

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Current Molecular-Based Detection Approaches for Waterborne *Cryptosporidium/Giardia*

1. **Species Identification and Genotyping**
   - Restriction Fragment Length Polymorphism (RFLP)-DNA Sequence Analysis
   - Single Strand Conformational Polymorphism (SSCP)
   - Randomly Amplified Polymorphic DNA (RAPD)
   - Multi-Locus Sequence Typing (MLST)

2. **Quantitative PCR**
   - Real-Time PCR (qPCR)
   - Loop-Mediated Isothermal Amplification PCR (LAMP)

3. **Viability Assays**
   - Reverse Transcriptase-PCR (RT-PCR)
   - Integrated Cell Culture/PCR
   - Nucleic Acid-Based Sequence Amplification (NASBA)
   - Fluorescence In Situ Hybridization (FISH)

4. **Microarray**

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Tools for Source Tracking, Species Identification, and Genotyping

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Biohazard Assessment Research Branch

Tracking Sources of Contamination in a Watershed

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2008, p. 6495–6504 Vol. 74, No. 21
0099-2240/08/$08.00+0 doi:10.1128/AEM.01345-08

Cryptosporidium Source Tracking in the Potomac River Watershed∇

Wenli Yang,[1] Plato Chen,[2] Eric N. Villegas,[3] Ronald B. Landy,[4] Charles Kanetsky,[4] Vitaliano Cama,[1] Theresa Dearen,[1] Cherie L. Schultz,[5] Kenneth G. Orndorff,[6] Gregory J. Prelewicz,[7] Miranda H. Brown,[8] Kim Roy Young,[4] and Lihua Xiao[1]*

Centers for Disease Control and Prevention, Atlanta, Georgia 30341[1]; Washington Suburban Sanitary Commission, Laurel, Maryland 20705[2]; National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 452683[3]; EPA Region III, Fort Meade, Maryland 20755[4]; Interstate Commission for the Potomac River Basin, Rockville, Maryland 20850[5]; Frederick County Division of Utilities and Solid Waste Management, Frederick, Maryland 21704[6]; Fairfax Water, Fairfax, Virginia 22031[7]; and Washington Aqueduct, Washington, DC 20016[8]

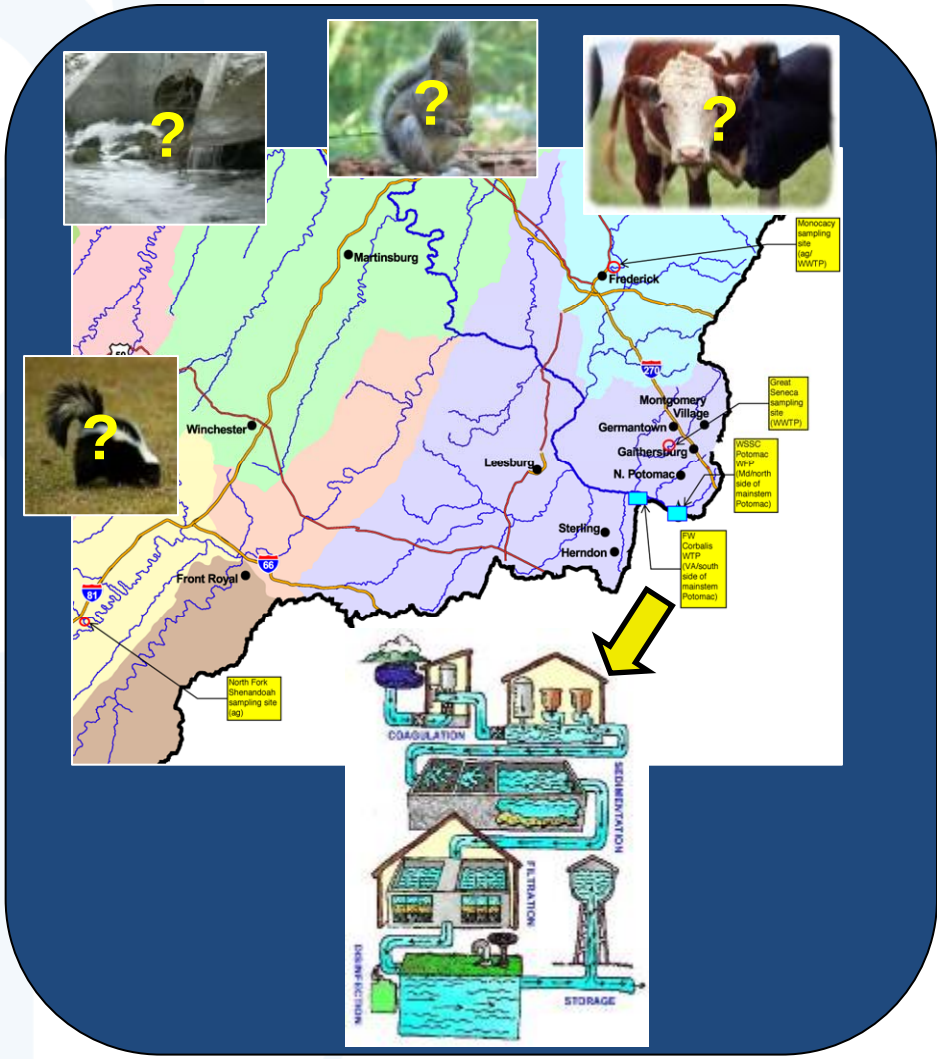
Received 16 June 2008/Accepted 22 August 2008

Goals

- Identify types of *Cryptosporidium* oocysts present
- Use PCR-RFLP and Method 1623
- Identify potential sources of *Cryptosporidium* oocysts in the Potomac River

Potential Sources:

Storm water runoffs
Wastewater treatment discharges
Wild animals
Agricultural/animal operations



Methodology

Collection of 20-L water samples (93 samples)

Filtration of two 10-L samples

Method 1623

One filter to an LT2 certified laboratory

Immunomagnetic separation of oocysts

Microscopy

One filter to CDC laboratory

Immunomagnetic separation of oocysts

PCR, DNA sequencing

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Cryptosporidium Species and Genotypes Found

TABLE 5. *Cryptosporidium* genotypes found in water samples in the Potomac watershed

Species or genotype	Major known host(s)	Minor known host(s)	No. of samples positive	No. of detections[a]	Detection site(s)
C. andersoni	Cattle	Sheep, humans (?)	41	167 (151 type A, 14 type B, and 2 type C sequences)	All except Great Seneca Creek[b]
C. felis	Cats	Cattle, humans	2	3	Great Seneca Creek
C. meleagridis	Birds	Humans, dogs, deer mice, brown rats	1	1	Great Seneca Creek
C. serpentis	Snakes, lizards		1	1	Potomac WFP
Deer mouse genotype III (W1)	Deer mice	Squirrels	3	5	Great Seneca Creek, Potomac WFP, Corbalis WTP
Deer mouse genotype IV (W3)	Deer mice		1	1	Great Seneca Creek
Cervine genotype (W4)	Sheep, zoo and wild ruminants, squirrels, chipmunks, woodchucks	Deer mice, beavers, raccoons, lemurs, humans	3	5	Great Seneca Creek
Muskrat genotype I (W7)	Muskrats, voles		3	4	Corbalis WTP, North Fork Shenandoah River, Monocacy River
Snake genotype (W11)	Snakes		1	1	Potomac WFP
W12			1	1	Great Seneca Creek
Skunk genotype (W13)	Skunks	Raccoons, otters, opossums, squirrels, humans	4	5	Great Seneca Creek, Potomac WFP, Corbalis WTP
Vole genotype (W15)	Voles		1	1	North Fork Shenandoah River
Tortoise genotype	Tortoises		1	1	Great Seneca Creek
C. bovis-like genotype			1	1	Potomac WFP
Mouse genotype II-like	Mice		1	3	North Fork Shenandoah River

[a] Total number of positive samples for five PCR replicates of all samples.
[b] Detected in one PCR replicate of one storm flow water sample from the Great Seneca Creek.

Yang, et.al. 2008. Applied and Environmental Microbiology

Summary

- *C. andersoni, a* cattle specific species, was the predominant oocyst detected at all sites tested
- Pathogenic *C. hominis* and *C. parvum* were not detected in all 93 samples analyzed
- Only minor species/genotypes infecting humans were detected (10 samples)
- *Giardia* data: 12 samples positive for *Giardia* cysts (1-50 cysts/10L). No molecular data available yet
- Molecular-based detection technique used in this project proves to be effective and sensitive to detect and genotype oocysts in source waters
- Study required 2 split samples; 1) Method 1623 and 2) Molecular genotyping…Expensive!
- Other source tracking studies done in other regions revealed similar results

Modifying Method 1623: Off-the-Slide Molecular Genotyping

Applied and Environmental Microbiology, Dec. 2005, p. 8991–8994 Vol. 71, No. 12
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.12.8991–8994.2005

Molecular Forensic Profiling of *Cryptosporidium* Species and Genotypes in Raw Water

Norma J. Ruecker,[1] Niravanh Bounsombath,[1] Peter Wallis,[2] Corinne S. L. Ong,[3,4] Judith L. Isaac-Renton,[3,4] and Norman F. Neumann[1,3]*

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Journal of Clinical Microbiology, Sept. 2006, p. 3285–3291 Vol. 44, No. 9
0095-1137/06/$08.00+0 doi:10.1128/JCM.00541-06

Rapid and Sensitive Detection of Single *Cryptosporidium* Oocysts from Archived Glass Slides

O. Sunnotel,[1] W. J. Snelling,[1] L. Xiao,[2] K. Moule,[3] J. E. Moore,[4] B. Cherie Millar,[4] J. S. G. Dooley,[1] and C. J. Lowery[1]*

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Applied and Environmental Microbiology, Aug. 2006, p. 5428–5435 Vol. 72, No. 8
0099-2240/06/$08.00+0 doi:10.1128/AEM.02906-05

Molecular Fingerprinting of *Cryptosporidium* Oocysts Isolated during Water Monitoring

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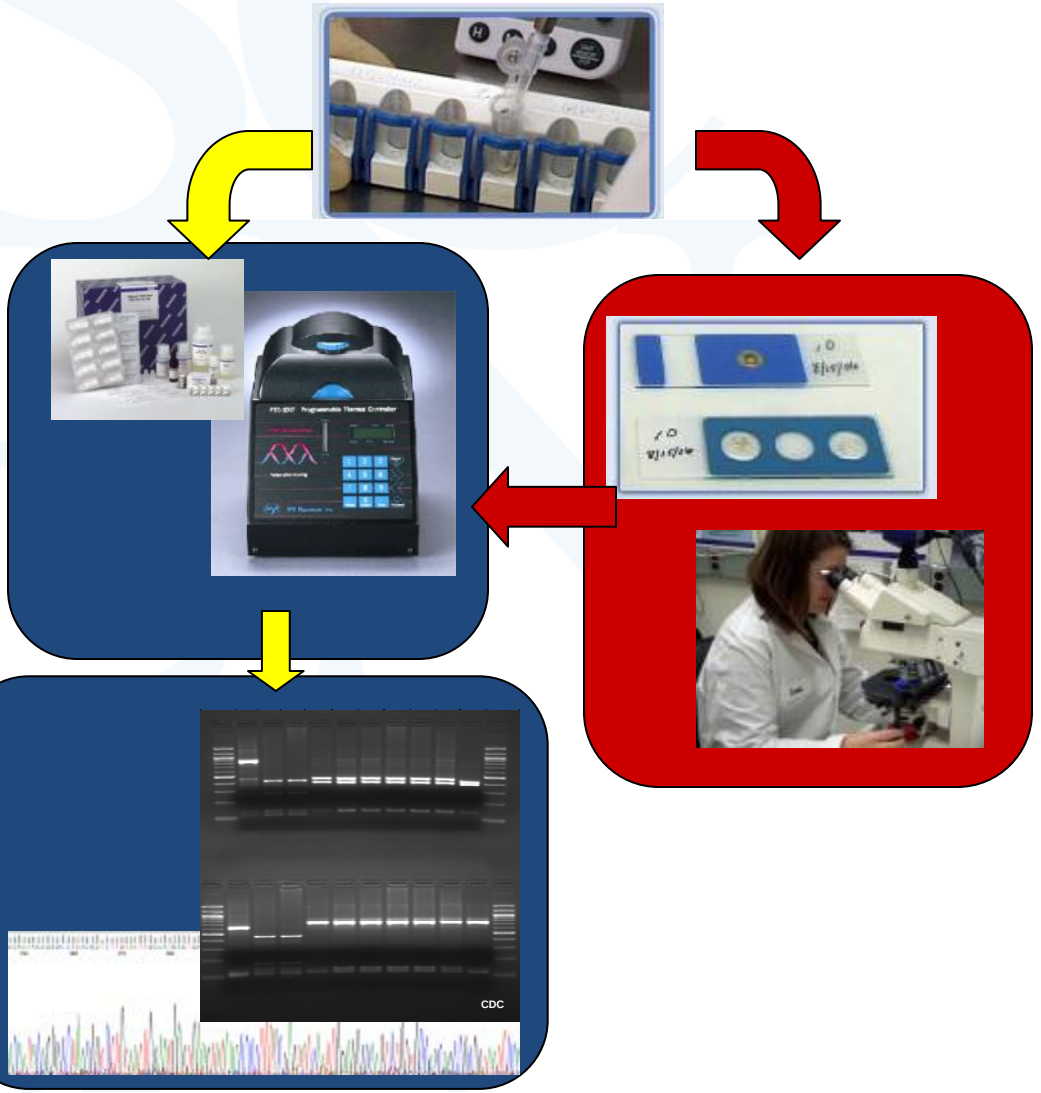
Applied and Environmental Microbiology, June 2007, p. 3945–3957 Vol. 73, No. 12
0099-2240/07/$08.00+0 doi:10.1128/AEM.02788-06

Tracking Host Sources of *Cryptosporidium* spp. in Raw Water for Improved Health Risk Assessment∇

Norma J. Ruecker,[1,2] Shannon L. Braithwaite,[2] Edward Topp,[3] Thomas Edge,[4] David R. Lapen,[5] Graham Wilkes,[5] Will Robertson,[6] Diane Medeiros,[6] Christoph W. Sensen,[7] and Norman F. Neumann[1,2]*

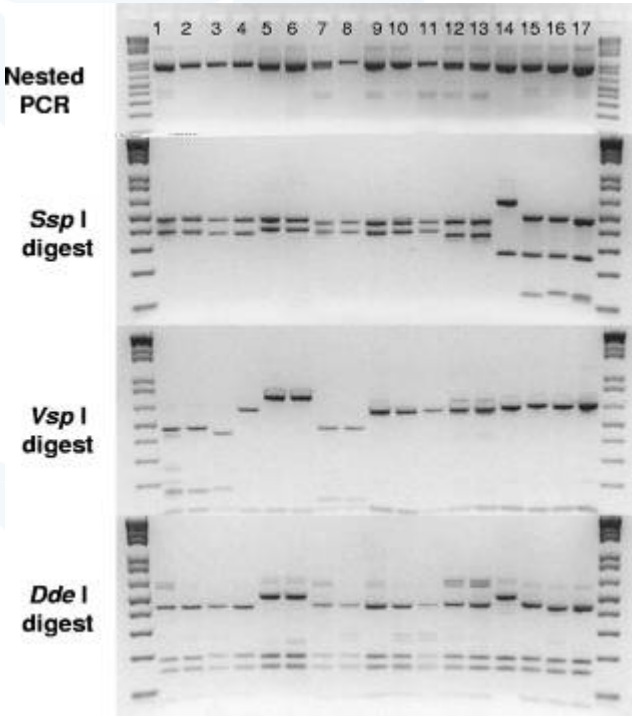
Alberta Provincial Laboratory for Public Health (Microbiology), Calgary, Alberta, Canada[1]; Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada[2]; Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, London, Ontario, Canada[3]; National Water Research Institute, Burlington, Ontario, Canada[4]; Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada[5]; Water Quality and Health Bureau, Health Canada, Ottawa, Ontario, Canada[6]; and Department of Biochemistry and Molecular Biology, Sun Center of Excellence for Visual Genomics, University of Calgary, Calgary, Alberta, Canada[7]

Received 29 November 2006/Accepted 23 April 2007



Off-the-Slide Molecular Genotyping

- Representative genotypes detected:
  - *C. andersoni*
  - *C. baileyi*
  - *C. parvum*
  - *Cryptosporidium* muskrat genotype I/II
  - cervine genotype
  - *Cryptosporidium* fox genotype,
  - genotype W1 and W12
- Average *Cryptosporidium* oocyst levels detected:
  - 0.09-0.26 oocysts/L



Reucker, N. et. al. 2007. AEM. 37:3945-3957

Summary

- Genotyping approaches suggest Method 1623 can overestimate levels of pathogenic species of *Cryptosporidium* detected in the watershed
- Off-the-slide genotyping offers post-microscopic genotyping
- Molecular-based detection assays are useful for tracking sources of *Cryptosporidium/Giardia* contamination
- Potential application for round 2 of LT2 in 2015
- Off-the-slide genotyping is still labor intensive and requires extensive technical experience

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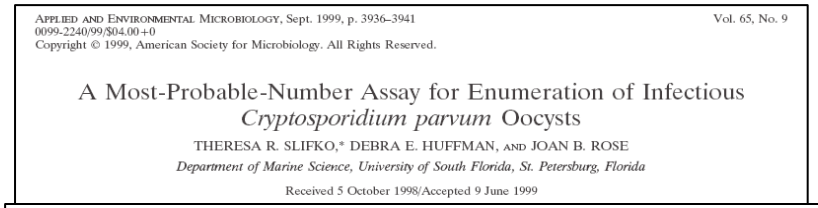
Tools for Detecting Viable/Infectious *Cryptosporidium* and *Giardia*

Detecting Viable/Infectious *Cryptosporidium* and *Giardia*


APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1999, p. 3936–3941 Vol. 65, No. 9
0099-2240/99/$04.00+0
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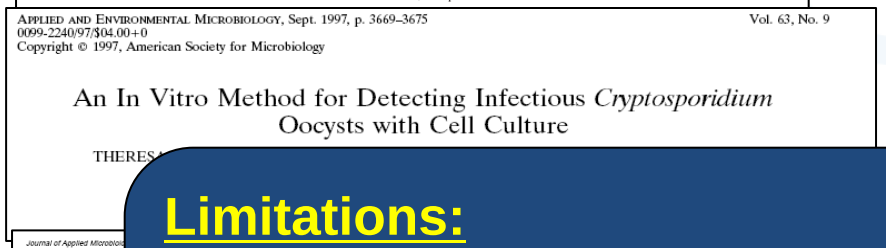
A Most-Probable-Number Assay for Enumeration of Infectious *Cryptosporidium parvum* Oocysts

THERESA R. SLIFKO,* DEBRA E. HUFFMAN, AND JOAN B. ROSE

Department of Marine Science, University of South Florida, St. Petersburg, Florida

Received 5 October 1998/Accepted 9 June 1999


APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1997, p. 3669–3675 Vol. 63, No. 9
0099-2240/97/$04.00+0
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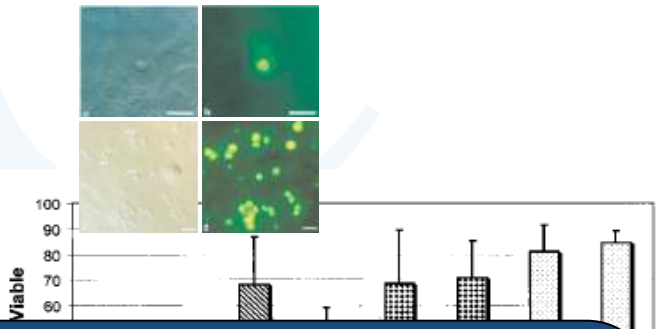
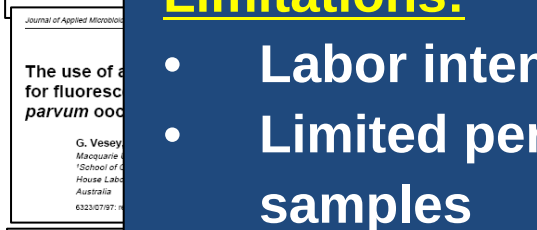
An In Vitro Method for Detecting Infectious *Cryptosporidium* Oocysts with Cell Culture

THERES



Limitations:

- **Labor intensive and expensive**
- **Limited performance evaluation data on environmental samples**
- **Limited range of species/genotypes detected**


Journal of Applied Microbiology

The use of a
for fluoresc
parvum ooc

G. Vesey
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House Lab
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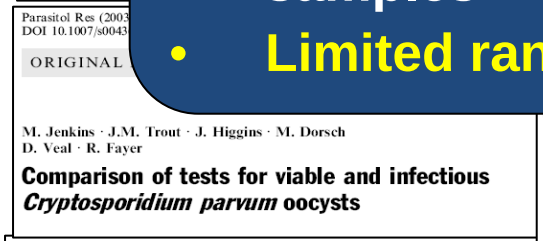
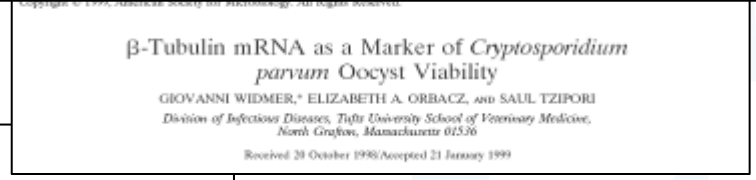
6323/07/97:


Parasitol Res (2003
DOI 10.1007/s0043

ORIGINAL

M. Jenkins · J.M. Trout · J. Higgins · M. Dorsch
D. Veal · R. Fayer

Comparison of tests for viable and infectious *Cryptosporidium parvum* oocysts


β-Tubulin mRNA as a Marker of *Cryptosporidium parvum* Oocyst Viability

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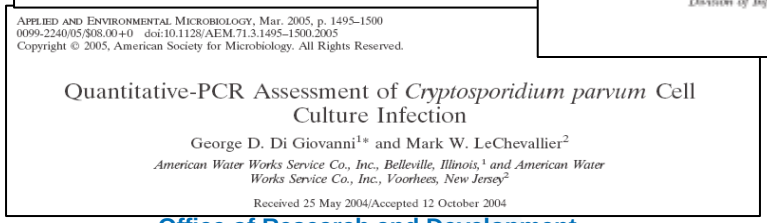
Division of Infectious Diseases, Tufts University School of Veterinary Medicine, North Grafton, Massachusetts 01536

Received 20 October 1998/Accepted 21 January 1999


APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2005, p. 1495–1500
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.3.1495–1500.2005
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Quantitative-PCR Assessment of *Cryptosporidium parvum* Cell Culture Infection

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American Water Works Service Co., Inc., Belleville, Illinois,[1] and American Water Works Service Co., Inc., Voorhees, New Jersey[2]

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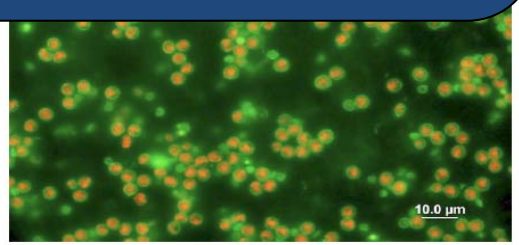

Fig. 1. FISH of *C. parvum* oocysts with Cy3-labelled 26-mer SSU rDNA oligonucleotide probe, Cpar677. Positive hybridisation was revealed by bright red fluorescence within oocysts. Oocysts were counterstained by immunolabelling (green) with CRY104-FITC (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Detection of Live/Viable *Cryptosporidium* Oocysts Using Propidium Monoazide (PMA)

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2007, p. 5111–5117 Vol. 73, No. 16
0099-2240/07/$08.00+0 doi:10.1128/AEM.02987-06

Use of Propidium Monoazide for Live/Dead Distinction in Microbial Ecology[▽]

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Center for Biofilm Engineering, Montana State University, Bozeman, Montana 59717[1]; Universidad de Antofagasta, Antofagasta, Chile[2]; and Department of Civil Engineering, Montana State University, Bozeman, Montana 59717[3]

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Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells

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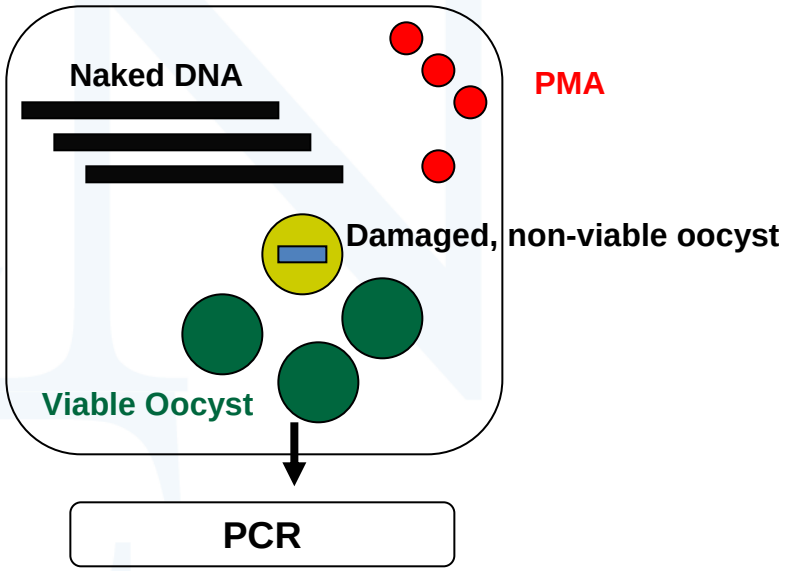
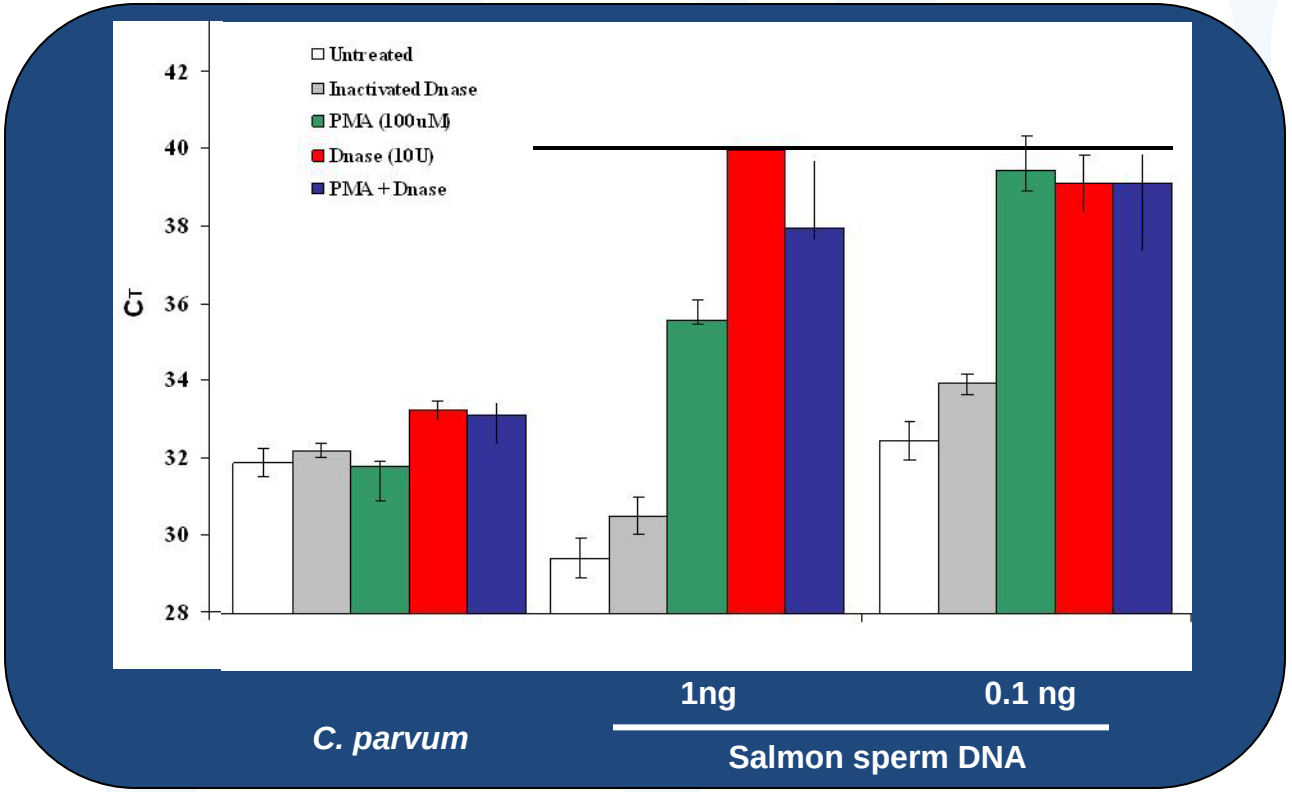


Removal of Indigenous DNA ("Naked DNA") Using Propidium Monoazide (PMA) and DNase

- Presence of indigenous DNA and dead oocysts in complex matrices
- Can affect outcome of PCR-based detection assays (overestimate)
- Detection of DNA vs. oocysts, how do we interpret results?
- Can we specifically detect and genotype "viable" pathogens in the water?



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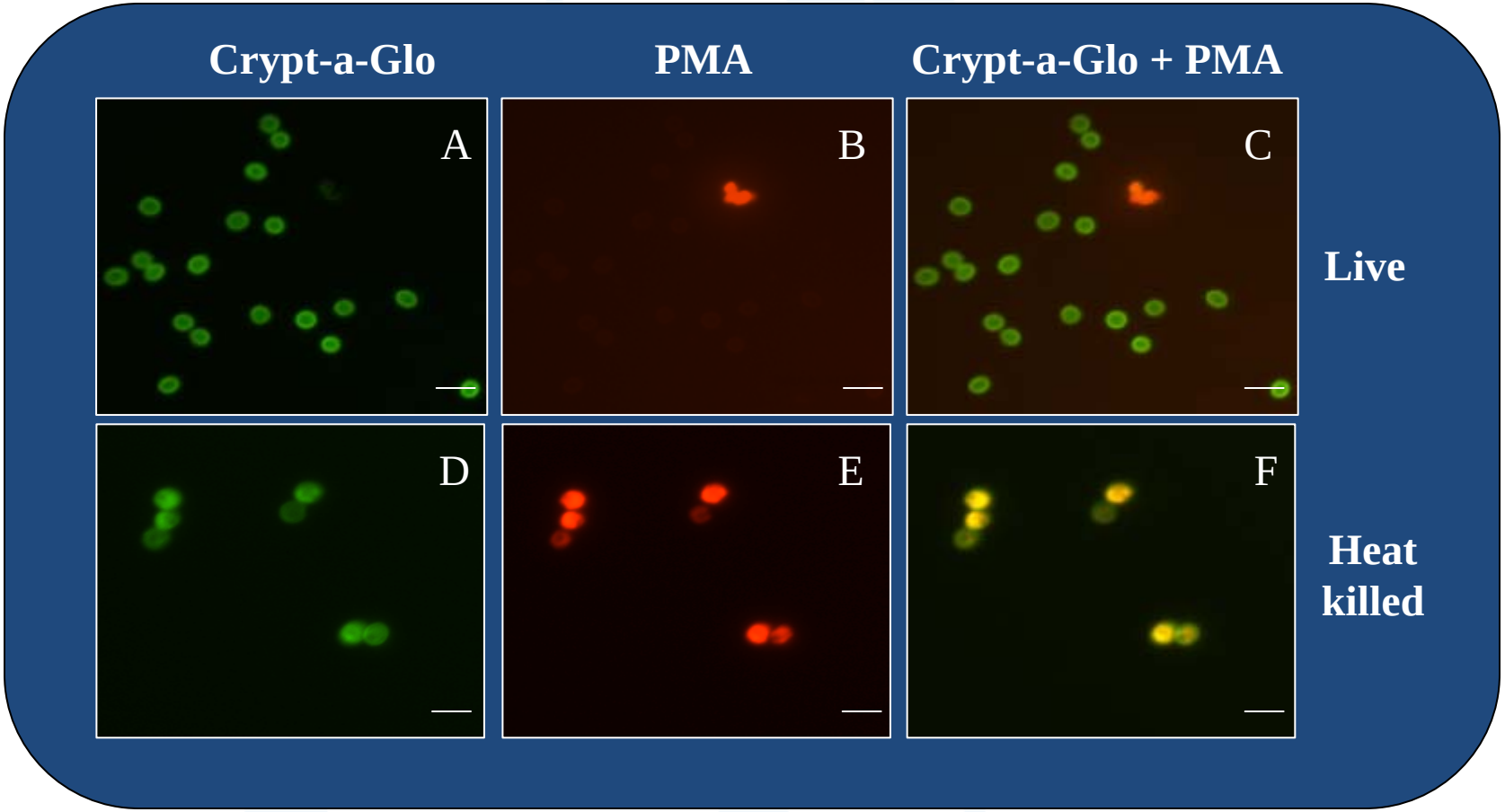
Detection of Live vs. Dead *C. parvum* Oocysts



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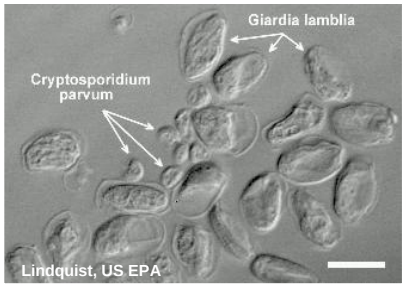
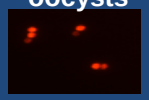
Detecting Live Oocysts Using PMA



PMA + Light

Dead/damaged oocysts



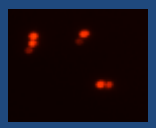
PCR-Genotyping

Oocyst		Ratio	Experiment			Live oocysts detected	Dead oocysts detected
Live	Heat-killed		1	2	3		
C. parvum	*C. muris*	0:100	-	-	-	0/3	0/3
C. parvum	*C. muris*	1:99	-	-	-	0/3	0/3
C. parvum	*C. muris*	10:90	Cp	-	Cp	2/3	0/3
C. parvum	*C. muris*	50:50	Cp	Cp	Cp	3/3	0/3
C. parvum	*C. muris*	100:0	Cp	Cp	Cp	3/3	0/3

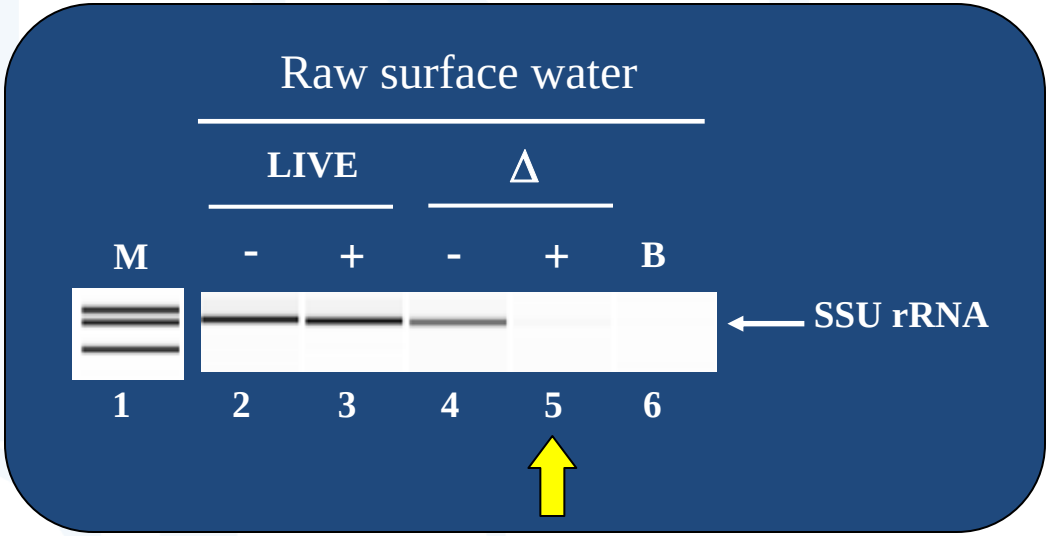
Is this Method Effective In Detecting Oocysts in Environmental Water Samples?

PMA + Light

Dead/damaged oocysts



PCR-Genotyping



M, Marker; B, Blank; -, No PMA; +, with PMA

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Summary

- **Heat inactivated oocysts treated with PMA were not detected, whereas live oocysts were detected by conventional PCR**
- **This method was effective for genotyping and allowed for the identification of only live oocysts**
- **Results suggest that this method may be applied to environmental water matrices (in certain situations)**
- **Use of PMA is also effective on more complex eukaryotic protozoa…in addition to bacteria and fungi**

EPA
United States
Environmental Protection
Agency

What Lies Ahead for the Waterborne Protozoan Research Program?

Multiple Pathogen Detection Systems "Pathogen Sequence Fingerprinting"

Using DNA microarrays to detect multiple pathogen threats in water

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0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.4.1817–1826.2002

Genotyping *Cryptosporidium parvum* with an *hsp70* Single-Nucleotide Polymorphism Microarray

Timothy M. Straub,[1]* Don S. Daly,[2] Sharon Wunshel,[2] Paul A. Rochelle,[3] Ricardo DeLeon,[3] and Darrell P. Chandler[1]

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0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.7.3262–3271.2004

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

Zheng Wang,* Gary J. Vora, and David A. Stenger

Center for Bio/Molecular Science & Engineering, Naval Research Laboratory, Washington, D.C. 20375

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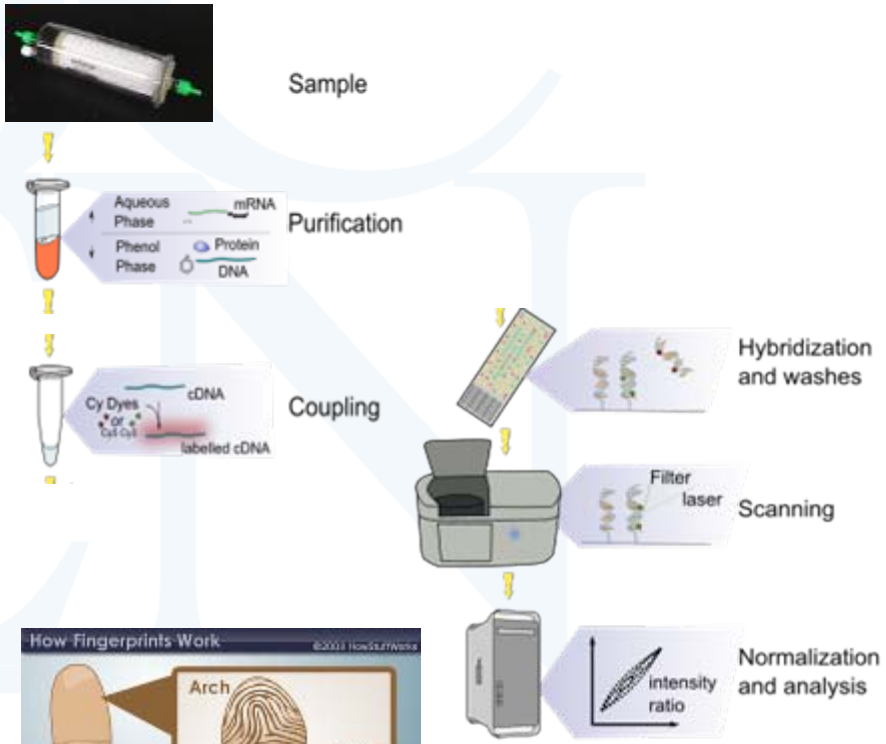
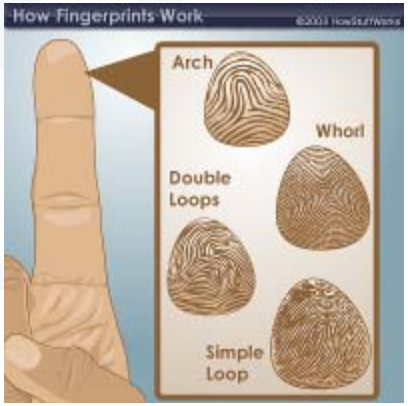
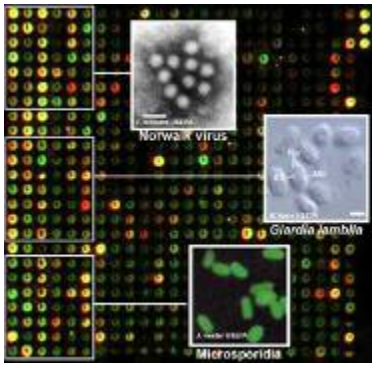






Molecular Detection Technologies: A Perspective

1. Molecular-based detection of *Cryptosporidium* and *Giardia* are in its infancy

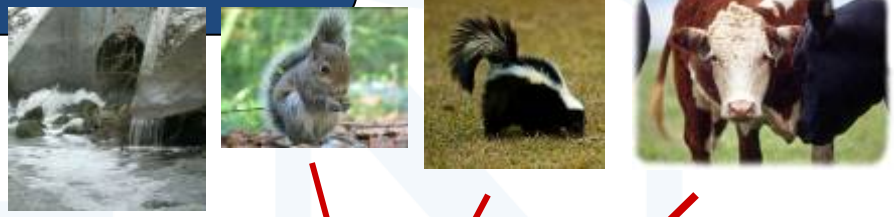
2. A better understanding of the differences between zoonotic and human-specific *Cryptosporidium/Giardia* is possible

3. Advances in the "Protozoan Detection Toolbox" will improve our understanding of these parasites and their relationship to public health

Using the Protozoan Detection Toolbox To Answer our Questions

United States Environmental Protection Agency

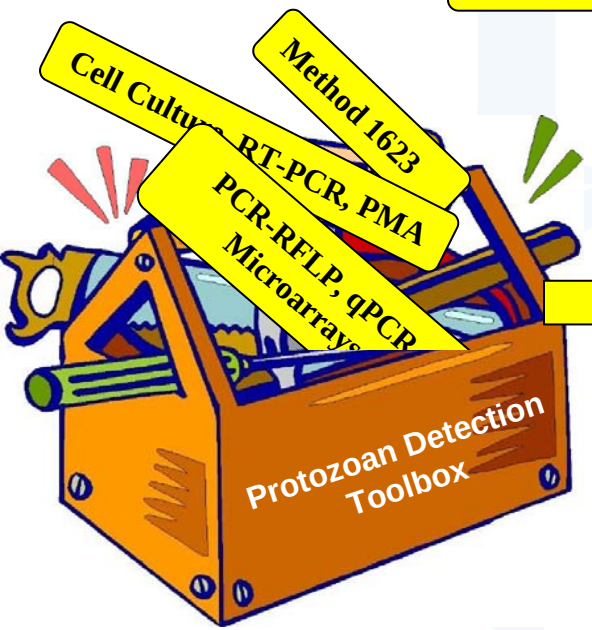
1. What are the total levels of *Cryptosporidium/Giardia* in the watershed?
2. What are the total levels of pathogenic *Cryptosporidium/Giardia* in the watershed?
3. How complex is the *Cryptosporidium/Giardia* species diversity in the watershed?
4. Are the *Cryptosporidium/Giardia* oocysts in the watershed viable/infectious?
5. Other questions…

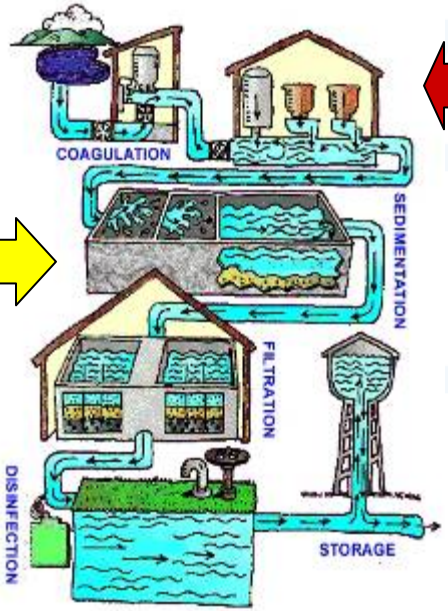


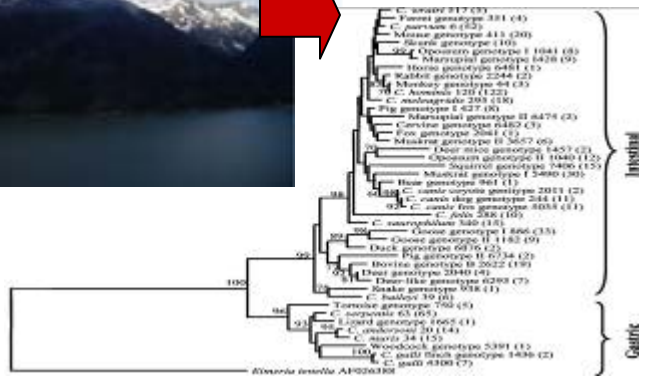
Method 1623

PCR-RFLP, qPCR, Microarrays

Cell Culture, RT-PCR, PMA







Pathogen ecology and source tracking

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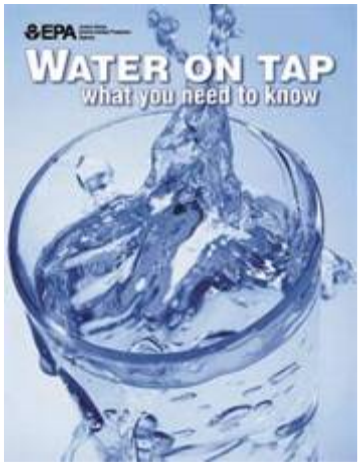
Interstate Commission for the Potomac River Basin
Cherie L. Schultz

United States Environmental Protection Agency



Questions?

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