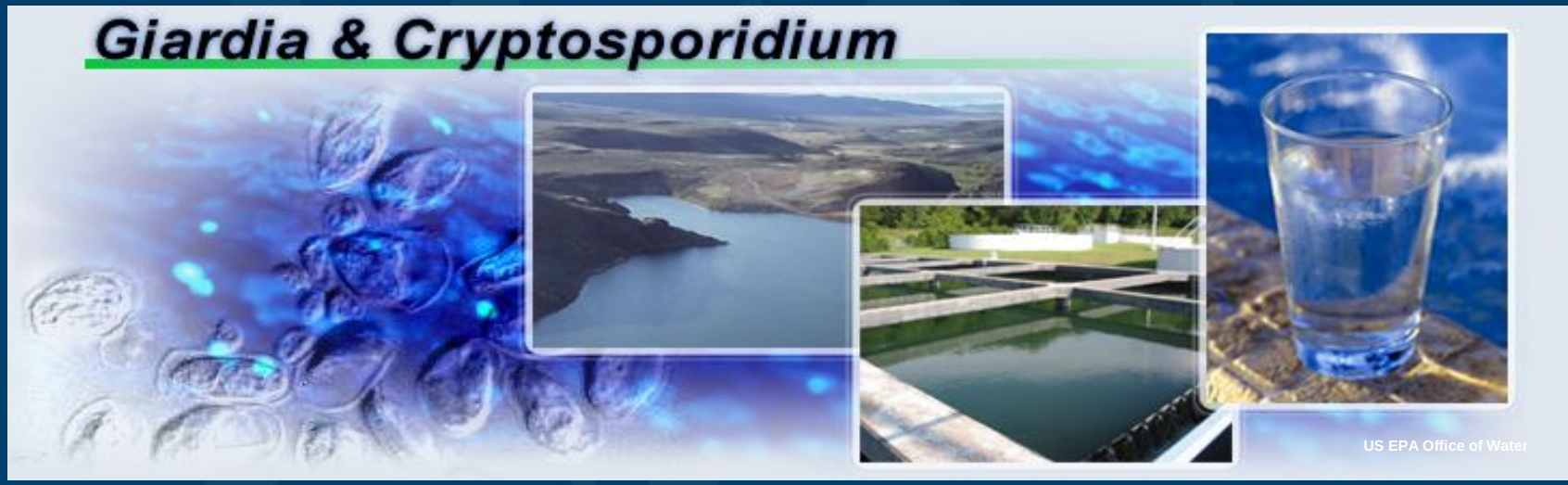
Surveillance Systems for Waterborne *Cryptosporidium*: Past, Present, and the Future



Eric N. Villegas, Ph.D.

2009 International Society of Exposure Science Annual Conference
Minneapolis, MN
1-5 November , 2009

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Overview

I. Brief introduction to waterborne *Cryptosporidium*

- **Historical perspective on detecting *Cryptosporidium***
- **Current detection methodologies**

II. US EPA's waterborne protozoan research program

- **Detecting, typing, and tracking sources of *Cryptosporidium* contamination**

III. The future of the "Microbial Detection Toolbox"

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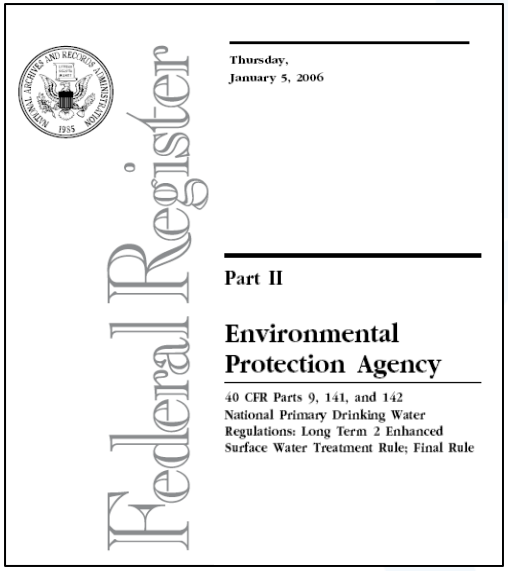
Cryptosporidium species

- **Waterborne enteric protozoan parasite**
- **Chronic diarrhea and death in susceptible groups**
- **1993 Milwaukee Outbreak**
- **Long Term 2 Enhanced Surface Water Treatment Rule (LT2) 2006**
- **20 valid species, with over 60 genotypes found**

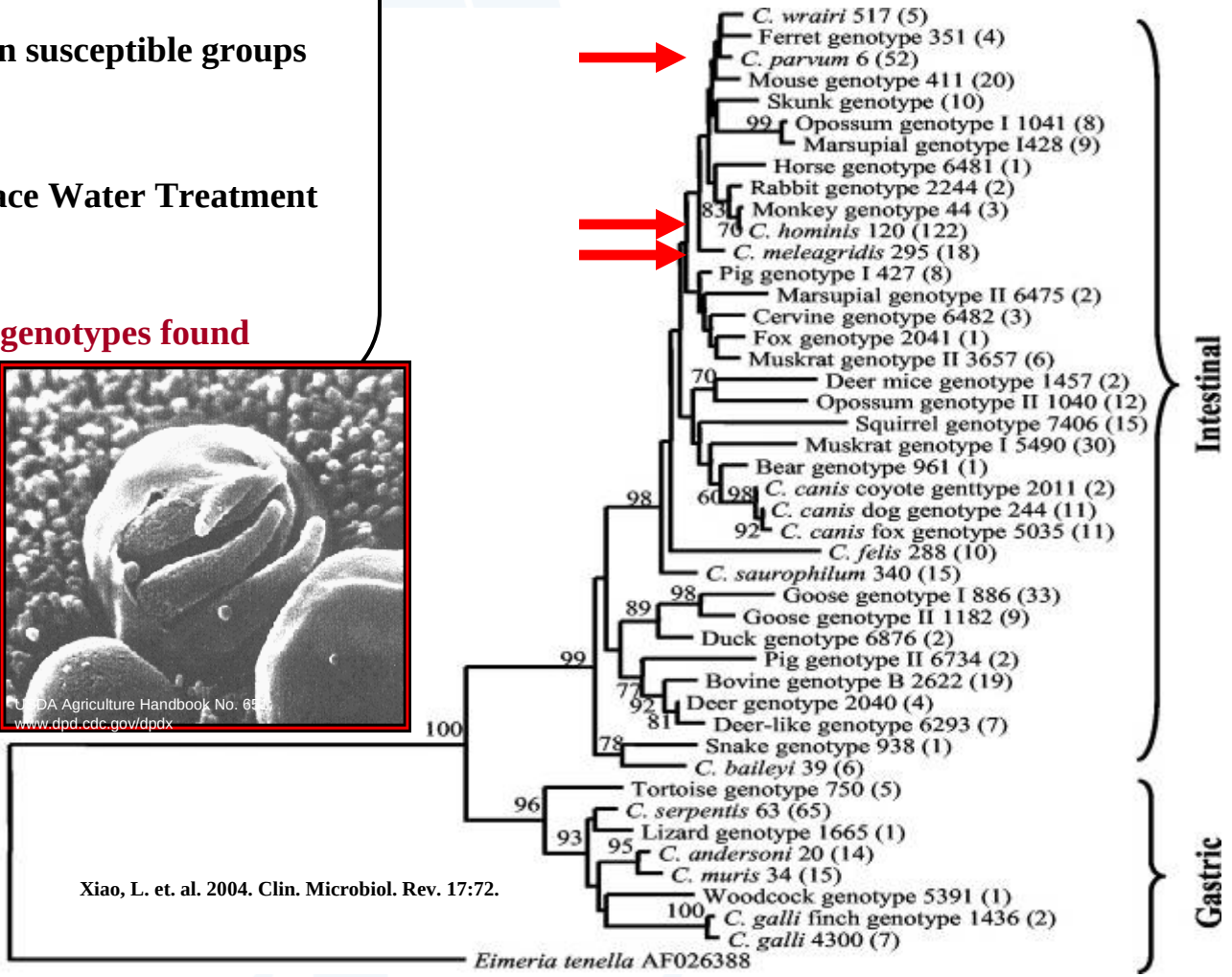

Federal Register

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



Xiao, L. et. al. 2004. Clin. Microbiol. Rev. 17:72.

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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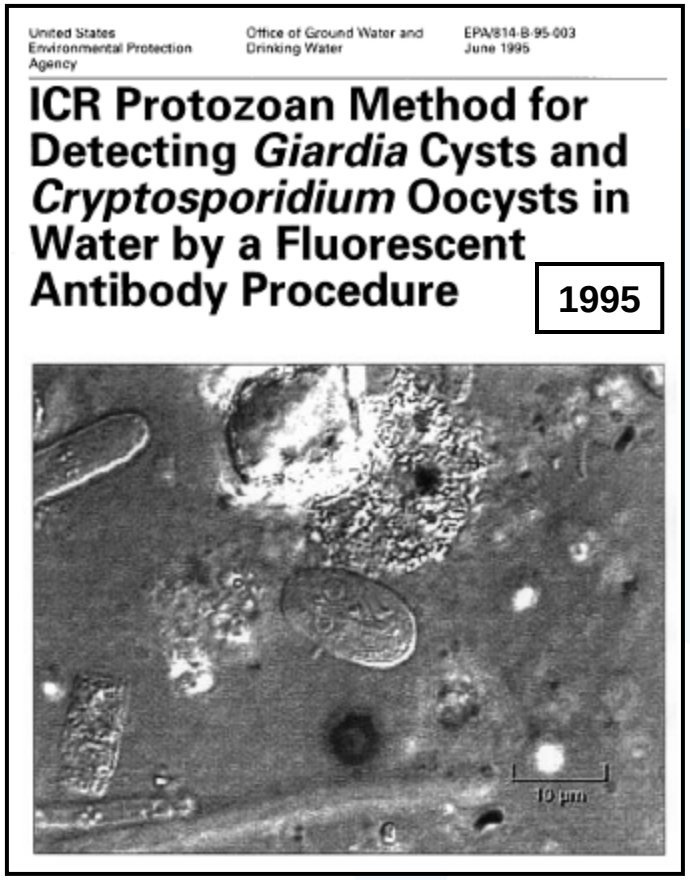
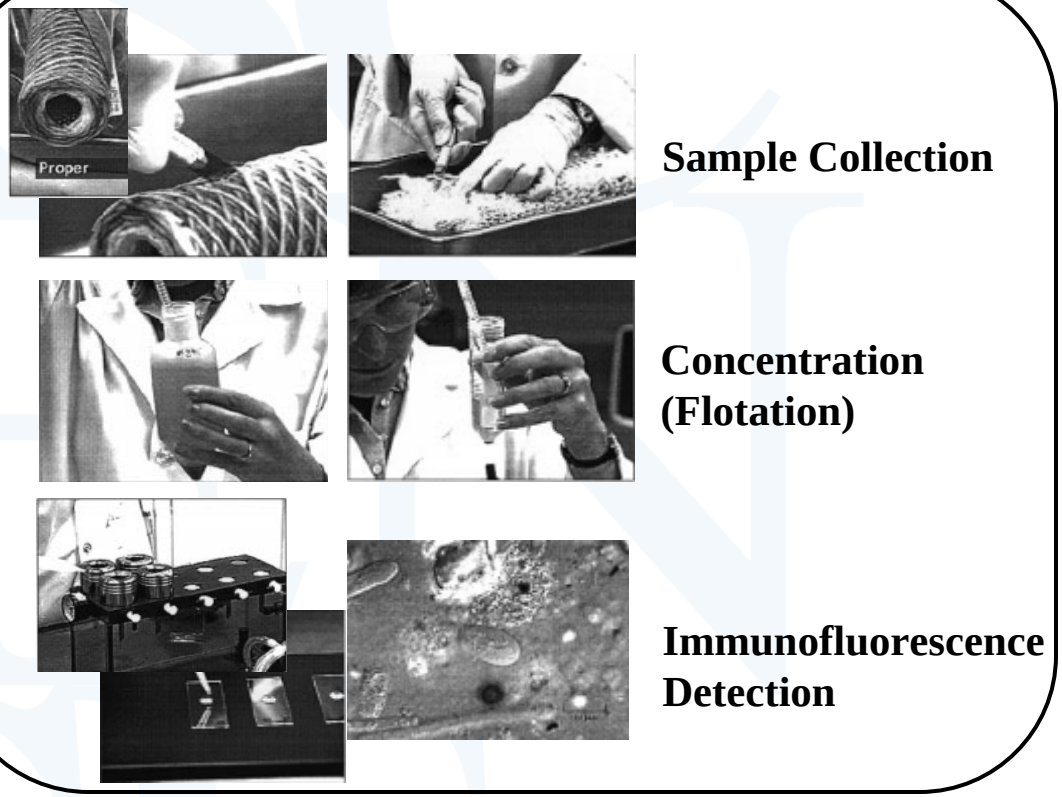
Detection of *Cryptosporidium* "The Past"


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

1995



Sample Collection

Concentration (Flotation)

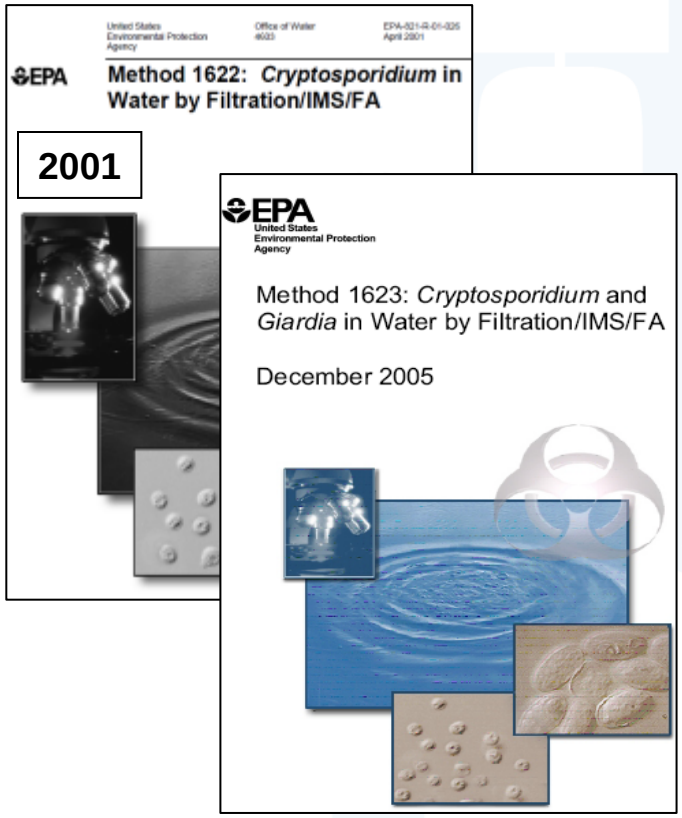
Immunofluorescence Detection

Limitations:

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

Detection of *Cryptosporidium* "The Present"

Method 1622/1623



Sample Collection

Concentration
(Immunomagnetic separation)

Immunofluorescence Detection

Limitations:

- **No species/genotyping capabilities**
- **No live vs. dead discrimination**

Challenges for the 21st Century
Developing a "Water Quality Tricorder"

Microbial Detection Systems:

1. Fast and user friendly
2. Sensitive and quantitative
3. Species/genotype specific
4. Live vs. dead
5. Identify source of contamination
6. Multiple organisms

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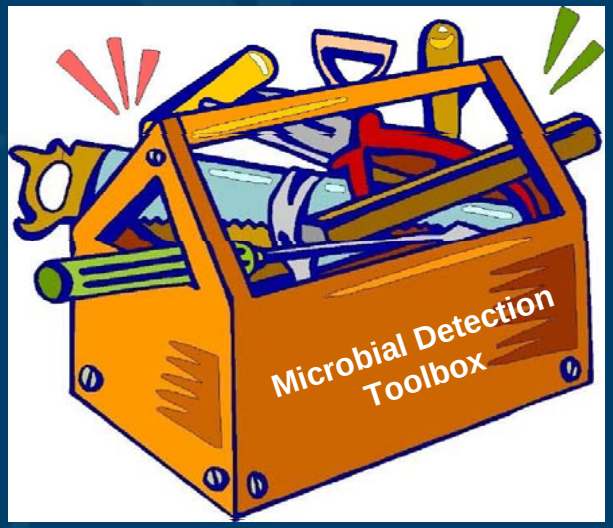
Advances in the Detection of *Cryptosporidium* Oocysts in Water

Building a "Microbial Detection Toolbox"



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Protozoan Detection Methods: Current Research Efforts

United States Environmental Protection Agency

I.

Collection/Filtration

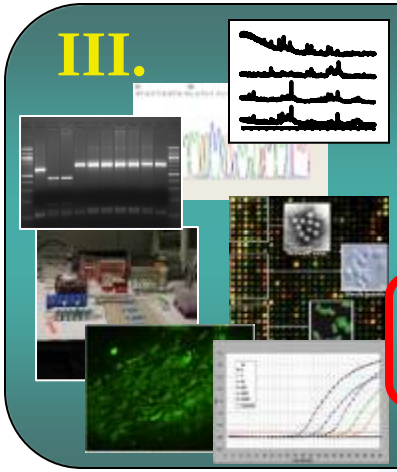
- Ultra filtration (single/multiple-pathogen)
- Continuous flow centrifugation (CFC)
- Membrane (charged) filters
- Glass wool

II.

Secondary Concentration

- Antibody-based (IMS)
- DEP/microfluidics
- Nanotechnology

III.



Detection Assays

- Microscopy-based (antibodies)
- *In vitro* cell culture and *in vivo* bioassays
- PCR-based assays
- Genomics and proteomics

All on-going and at various stages in development

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Detecting, Typing, and Tracking Sources of Human Infectious *Cryptosporidium* Oocysts in Water

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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
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Cryptosporidium Source Tracking in the Potomac River Watershed▽

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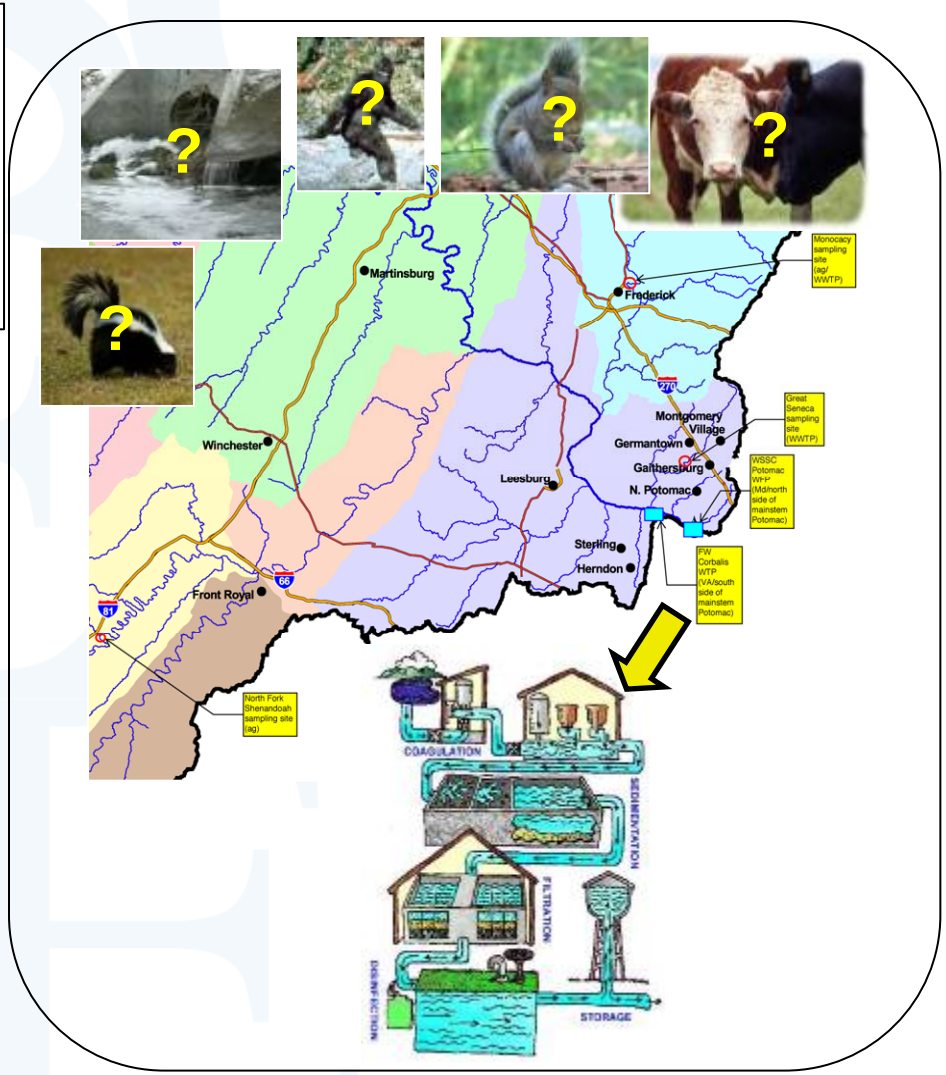
Received 16 June 2008/Accepted 22 August 2008

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



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

- **Predominant species detected: *C. andersoni*, not infectious to humans**
- ***C. hominis* and *C. parvum* not detected in all samples analyzed (93 samples)**
- **Only minor species/genotypes infecting humans were detected (10 samples)**
- **PCR is effective at detecting and typing oocysts contaminating the watershed**
- **Expensive! Required 2 split samples; 1) Method 1623 and 2) Molecular genotyping**
- **No live/dead differentiation**
- **Molecular source tracking data were useful towards improving management practices of the local water, agricultural, and live stock industries**

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How Can We Improve Current Genotyping Approaches?

By Adding a Live/Dead Selection Step…

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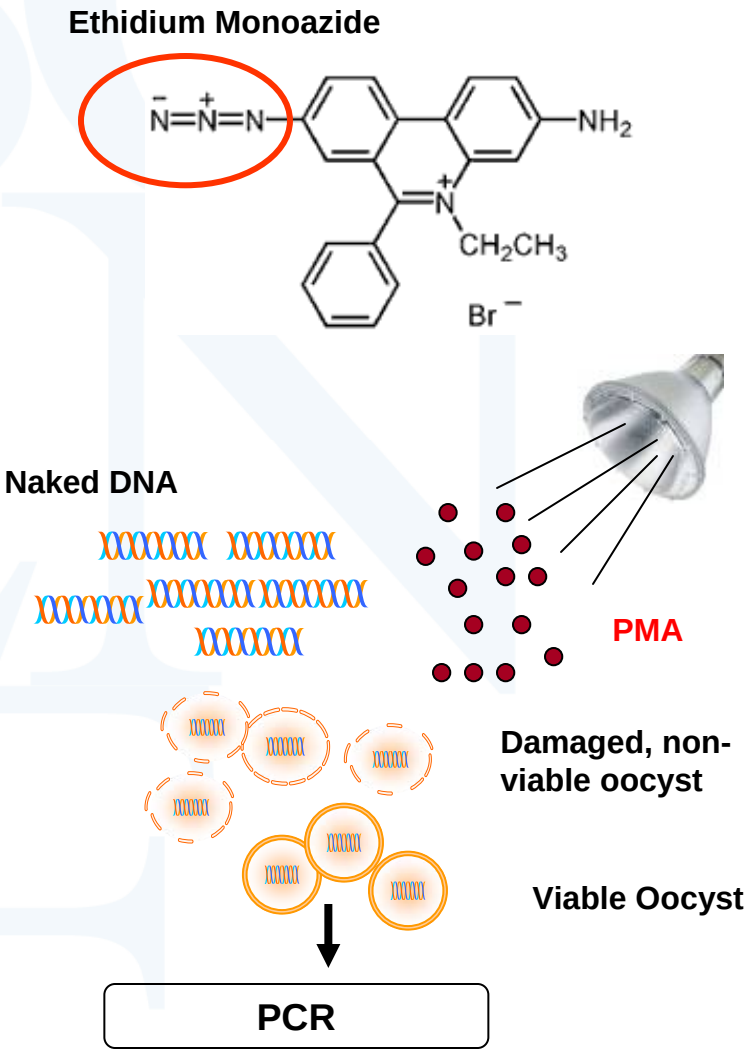
Using Propidium Monoazide (PMA) to Differentiate Live/Dead Organisms

- Selectively permeable to dead bacteria and fungi
- Intercalates into DNA and binds covalently to DNA upon light exposure
- Photolysis of the azide group produces a reactive nitrene radical which reacts to hydrocarbon moieties in nucleic acids
- PMA renders the DNA insoluble and subsequently removed during nucleic acid extraction procedures



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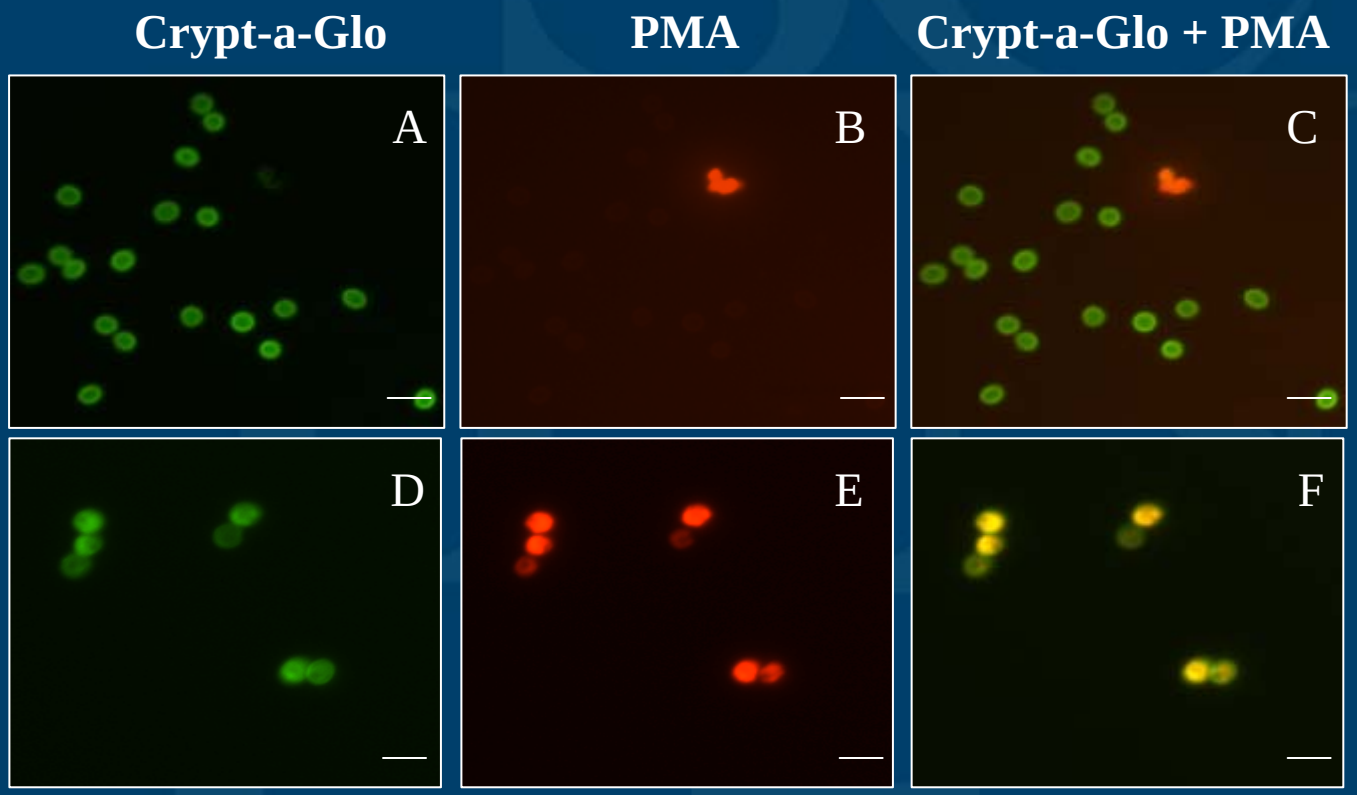
Detection of Live vs. Dead *C. parvum* Oocysts Using PMA



Brescia, et. al. 2009. Applied and Environmental Microbiology

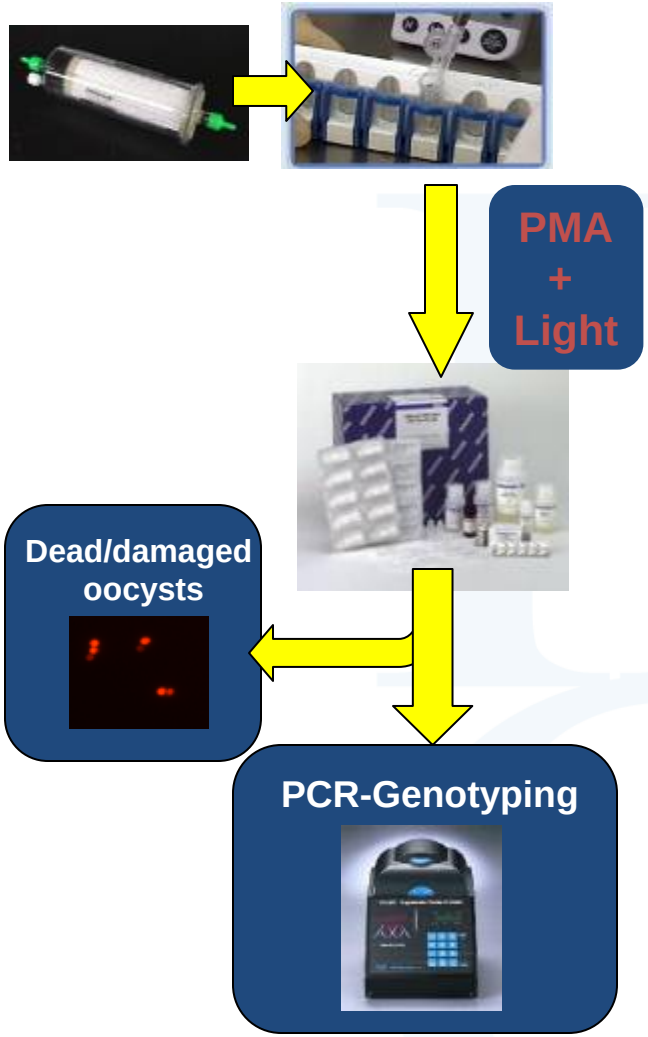
Molecular Genotyping of *C. muris* and *C. parvum* using CryptoPMA-PCR

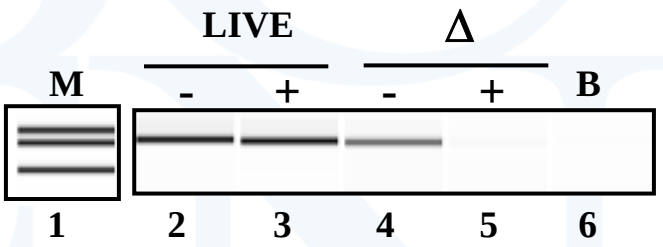
Oocyst		Ratio	Oocysts detected in experiments:			Live oocysts detected	Dead oocysts detected
Live	Heat-killed		1	2	3		
C. muris	*C. parvum*	0:100	-	-	-	0/3	0/3
C. muris	*C. parvum*	1:99	Cm	-	-	1/3	0/3
C. muris	*C. parvum*	10:90	Cm	Cm	Cm	3/3	0/3
C. muris	*C. parvum*	50:50	Cm	Cm	Cm	3/3	0/3
C. muris	*C.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

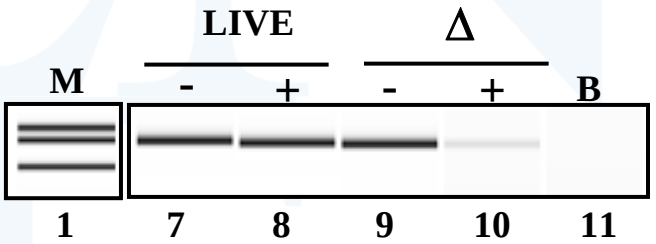
***SSU rRNA* and *hsp70* genes were amplified and sequenced**

Brescia, et. al. 2009. Applied and Environmental Microbiology

Is the CryptoPMA-PCR Effective in Detecting *Cryptosporidium* in Water?







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

Brescia, et. al. 2009. Applied and Environmental Microbiology

Summary

- **PMA pre-treatment allows for specific detection and genotyping of live *Cryptosporidium* oocysts;**
  - **By preventing amplification of naked DNA, old, damaged, and dead oocysts**
  - **Uses a culture-independent "viability" component**
  - **Results correlated with cell-culture assay**
- **Can potentially be incorporated into other molecular-based methods**
  - **qPCR, microarrays, etc.**
- **PMA has also been successfully applied to other microbes**
- **May be applied to evaluate "viable" oocysts present in environmental matrices (source or finished water)**

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What Lies Ahead for the "Microbial Detection Toolbox?

Advanced Molecular Detection Technologies: Real-time PCR, Microarrays, Whole Genome Sequencing, etc.

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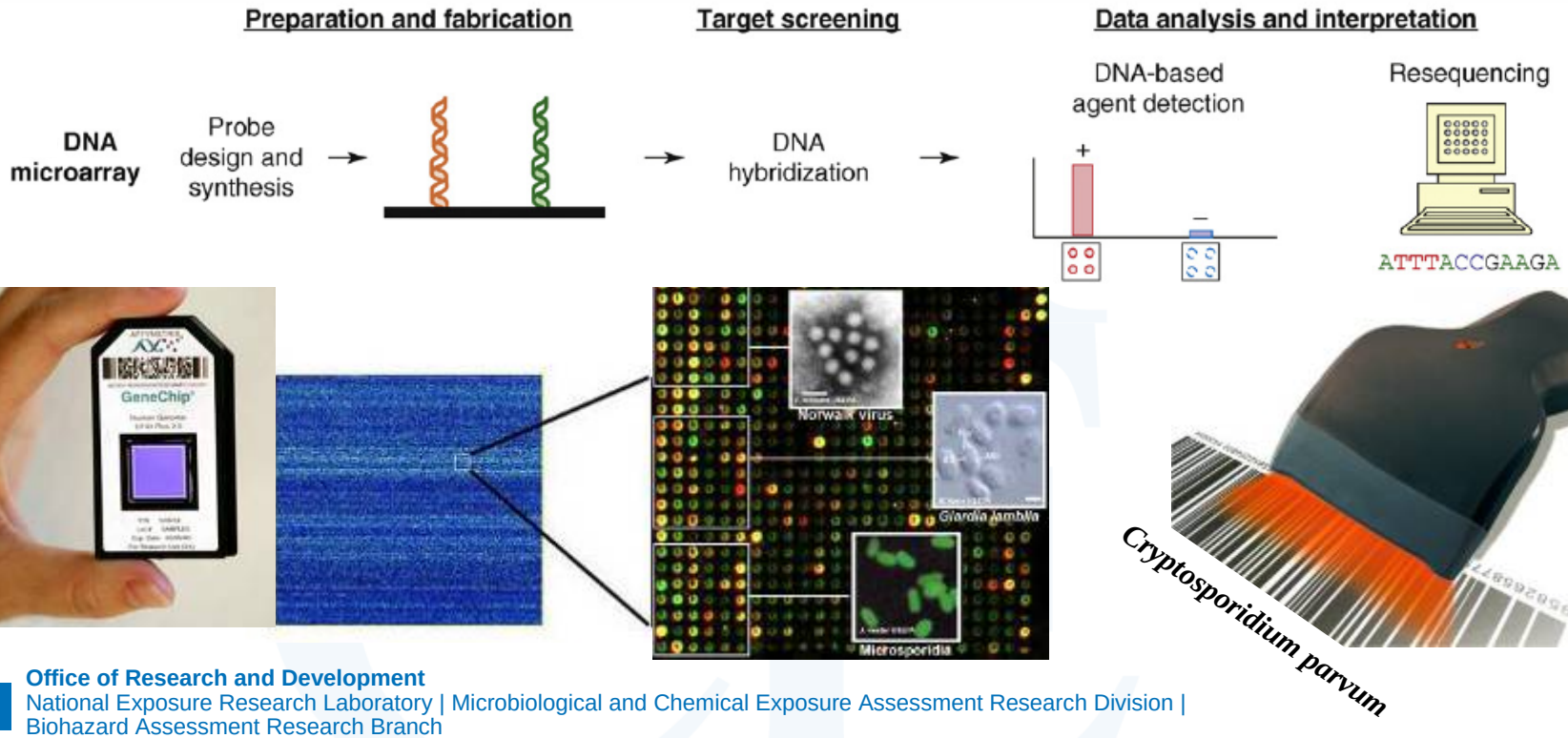
Microarrays for Detecting Multiple Pathogens "Pathogen Sequence Bar Coding"

Goal:

- **Identify unique DNA sequences from each organism of interest "bar codes"**
- **Use microarray technology to detect multiple pathogens:**
  - **bacteria, protozoa, and viruses**
- **Detect pathogens in environmental samples (water, soil, feces)**



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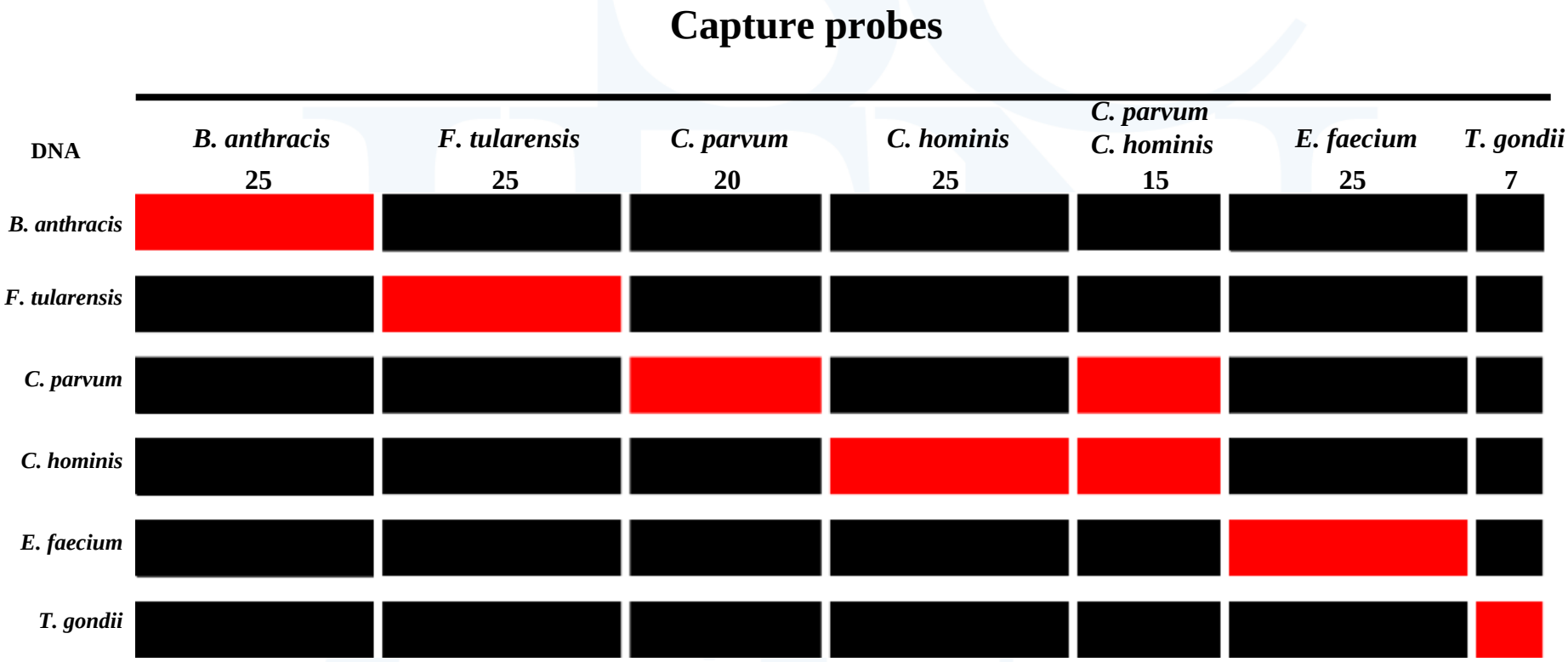
Microsequencing Array: Probe Specificity



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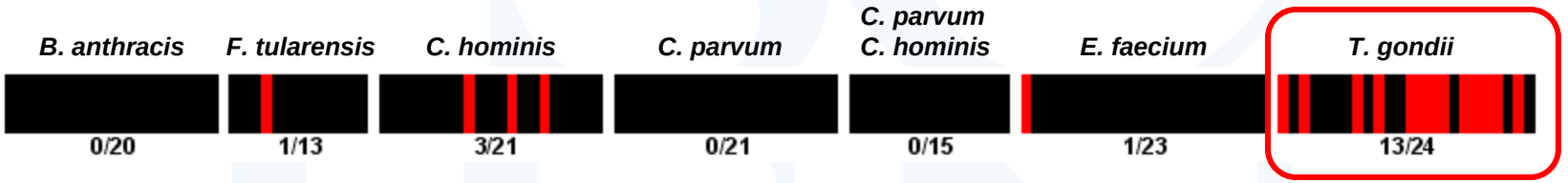
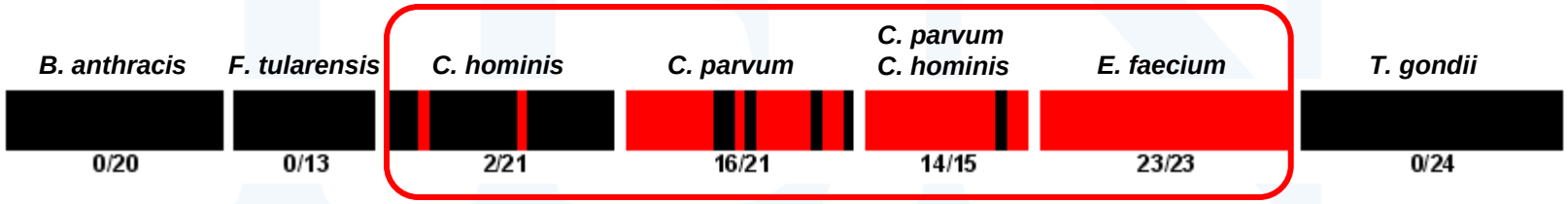
Brinkman, et. al. 2009. In preparation

Identification of Spiked Organisms in Tap Water Concentrates

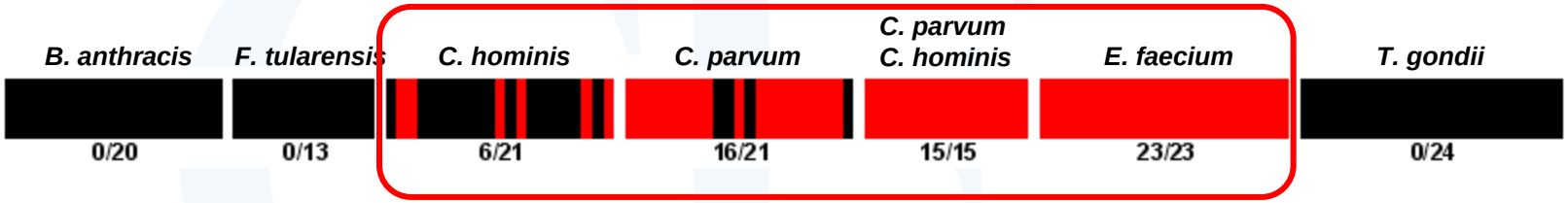
A. Reagent grade water: *G. lamblia, C. muris, E. coli K-12*, and *T. gondii*



B. Reagent grade water: *C. hominis, C. parvum, and E. faecium*



C. Tap water: *C. hominis, C. parvum, and E. faecium*



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Brinkman, et. al. 2009. In preparation

Outcomes and Potential Benefits

Summary:

- **Developed a microarray-based multiple pathogen detection and typing system**
- **Can only determine the presence or absence of the organism**
- **Works with tap water concentrates (Brinkman, et. al.2009. JVM)**
- **No viability component, but may be used with PMA**

Applications:

- **Multiple pathogen detection and typing system**
- **Potential to identify novel pathogens in the environment**
- **Microbial and pathogen source tracking studies**

Molecular Detection Technologies: A Perspective

1. Molecular-based detection of *Cryptosporidium* is still evolving

2. A better understanding of the differences between animal, zoonotic, and anthroponotic *Cryptosporidium* is possible

3. Continued advancements in the development of the "Microbial Detection Toolbox" will improve our understanding of risks associated with waterborne *Cryptosporidium* oocysts

Developing a "Water Quality Tricorder" Are We There Yet?

Microbial Detection Systems:

- √ 1. Fast and user friendly
- 2. Sensitive and quantitative
- √ 3. Species/genotype specific
- √ 4. Live vs. dead
- 5. Identify source of contamination
- √ 6. Multiple organisms

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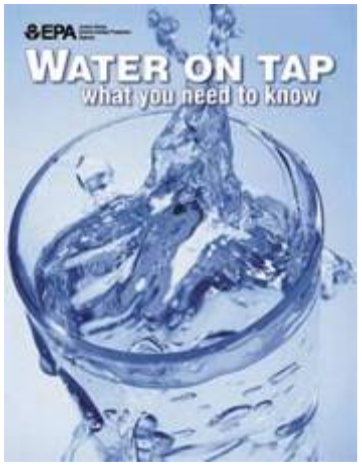

Questions?

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