JOHNS HOPKINS UNIVERSITY
CWH
Center for Water and Health

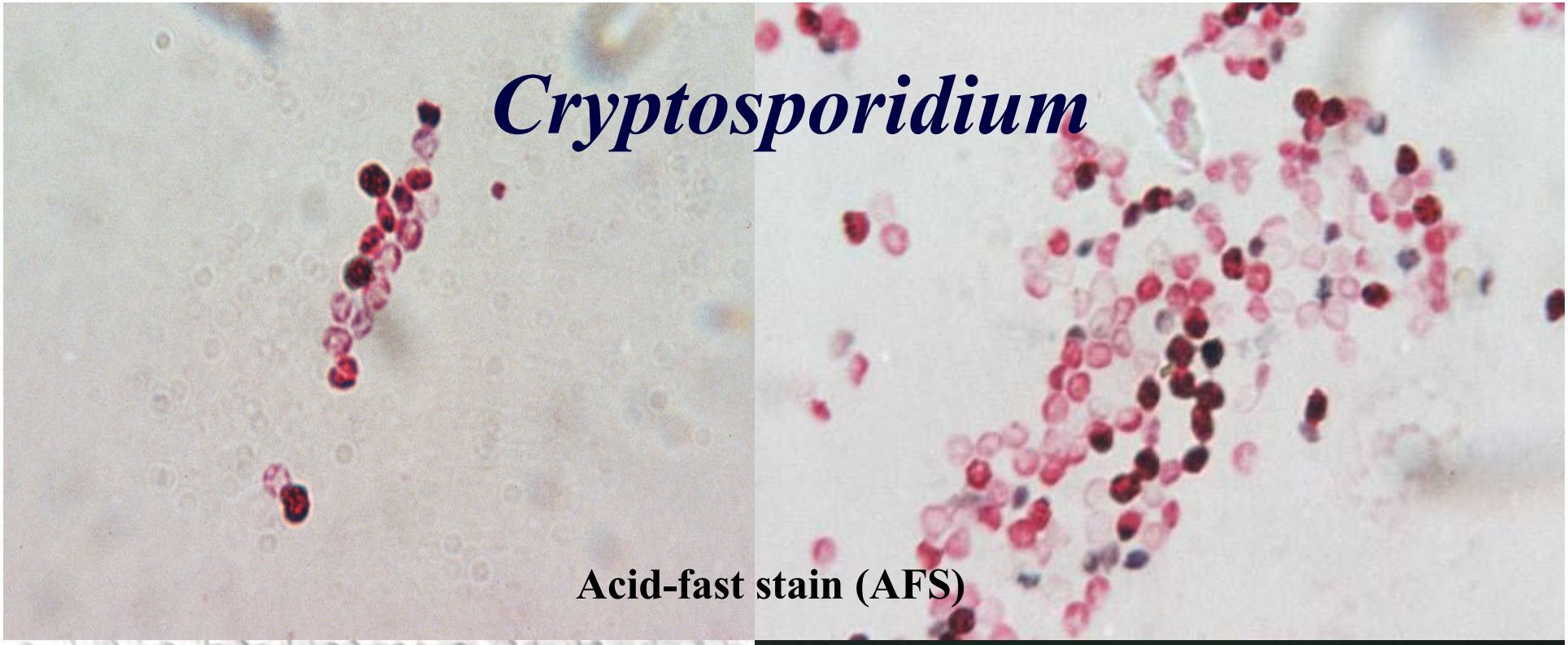
SAMPLING AND DETECTION METHODS

Thaddeus Graczyk

Department of Environmental Health Sciences, and Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

Cryptosporidium*, *Giardia*,** and human-infectious microsporidia, i.e., ***Encephalitozoon intestinalis, E. hellem, E. cuniculi, Enterocytozoon bieneusi

Cryptosporidium



Acid-fast stain (AFS)

Concentration

Species

Viability, Infectivity

Immunofluorescent Antibody (IFA)

Rose et al. 1997. Waterborne Cryptosporidiosis: Incidence, Outbreaks, and Treatment Strategies

Table 3 Studies on the Prevalence of *Cryptosporidium* in Surface and Groundwaters in North America

Water Source	Number of Samples	Average Positive (%)	Range	Geometric Average (oocysts/l)	Ref.
Stream/river[a]	6	100	0.80–5,800	1920[b]	36
Stream	19	73.7	0–240	1.09	34
Stream/river	58	77.6	0.04–18	0.94	32
Stream/river	38	73.7	<0.001–44	0.66	38
River[a]	11	100	2–112	25[c]	33
River/lake	85	87.1	0.07–484	2.70	39
River	22	31.8	0.01–75.7	0.58	39a
River/lake[a]	18	NA	7.1–28.5	17.8	35
Lake	20	70.7	0–22	0.58	34
Lake/reservoir	32	75.0	1.1–8.9	0.91	32
Lake	24	58.3	<0.001–3.8	1.03	38
Lake	44	27.3	0.11–251.7	4.74	39a
Pristine river	3	NA	NA	0.08	35
Pristine river	59	32.2	NA	0.29	38
Pristine lake	34	52.9	NA	0.093	38
Pristine spring	7	28.6	<0.003–0.13	0.04	38
Pristine lake	11	9.1	0–0.003	0.003	37
Well	18	5.6	NA	0.003[d]	38

Note: NA = information not available.

[a] Impacted by domestic and/or agricultural waste.
[b] Arithmetic mean.
[c] Data adjusted for recovery efficiencies.
[d] Single sample value.

33. Onghert and Stibbs 1987

35. Rose et al. 1988

39a. LeChevalier et al. 1991

1) Recovery of Oocysts from Water

Filtration, Continuous Flow Centrifugation, Centrifugation

2) Detection of Oocysts in Recovered Substrate

Elution, Dissolution, Concentration, Separation, Purification, Staining, Microscopic examination, Flow cytometry

- ~~Am Standards for Testing Materials Method (ASTM Standards)~~
- ~~Standard Method (AWWA Standards)~~
- ~~Alternate Method (Hansen & Ongerth, 1991)~~
- Cellulose Acetate Membrane (CAM)-Filter Dissolution Method (Graczyk et al., 1997)
- Method 1622 (U.S. EPA); Method 1623 (U.S. EPA)
  (limitations: 10L, drinking water (=low turbidity)

Recovery of Oocysts from Water

Detection of Oocysts in Recovered Substrate

Filtration*, Continuous Centrifugation, Flow Centrifugation

Elution*, Dissolution, Concentration, **IM Separation***, Purification, Flow Cytometry

Staining*, Examination*, Enumeration*

*** 1622, 1623**

CAM-Filter Dissolution Method vs Method 1623

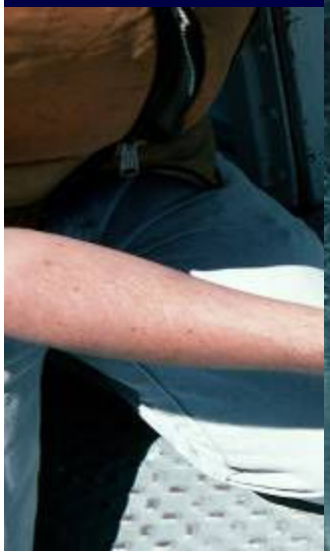
CAM-Filter Dissolution Method	Method 1623
Filtration	Filtration
Container Elution*	……
Filtration	……
Membrane Dissolution**	Membrane Elution
IM Separation	IM Separation
Staining	Staining
Examination	Examination
Enumeration	Enumeration

* Eluting fluid: SDS, Tween-80, antifoam agent

** Acetone treatment permeabilize C*ryptosporidium* oocysts which is necessary for the Fluorescent *In Situ* Hybridization (FISH) reaction

Cellulos n Method

2
0



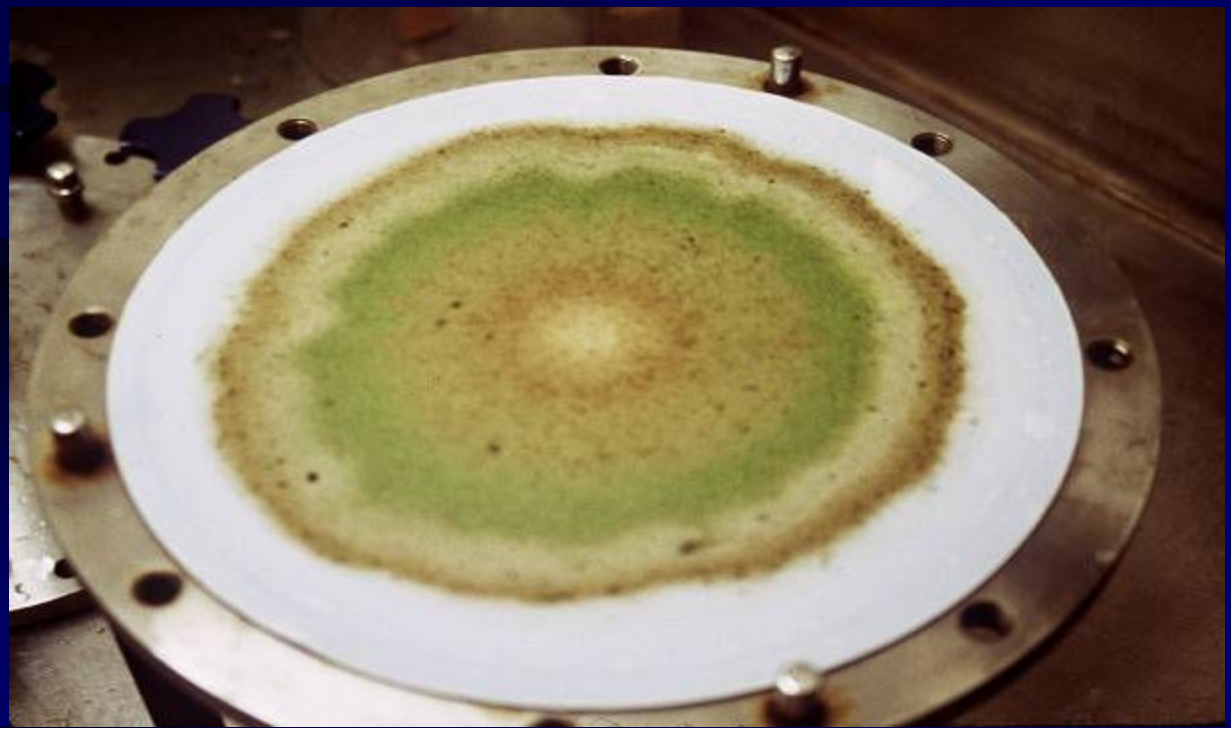
CAM-Filter Dissolution Method vs Method 1623

1) **Dissolution of the Membrane** vs **Elution of the Membrane**

   restoration of cellulose fibers residue left on the membrane

2) **Elution of the Container** vs **……**

   Graczyk et. al. (1997)



Graczyk et al. 1977. Recovery of waterborne oocysts of *Cryptosporidium* from water samples by the membrane filter dissolution method. *Parasitol Res* 83: 121-125.

Table 1 Recovery efficiency of *Cryptosporidium parvum* (AUCP-1 strain) oocysts seeded to 25 l of drinking water in the polyethylene carboy aspirator bottle by dissolution of a 25-mm-diameter, 1.2-µm-pore CAM. Each experiment was performed in quadruplicate

Seeded oocysts[a]			Mean number (%) of oocysts recovered		
Number	Concentration		From 25 l of water	Eluted after draining of the water	Total
	per liter	per cup[b]			
12,863	514	123	8,236 (64.0)	2,350 (18.3)	10,575 (82.3)
6,200	248	59	3,172 (51.2)	1,800 (29.0)	4,975 (80.2)
4,680	187	45	1,744 (37.3)	1,630 (34.8)	3,375 (72.1)
3,050	122	29	732 (24.0)	1,730 (56.7)	2,550 (80.7)

[a]Based on the enumeration from 15 ml of PBS eluting fluid
[b]Equal to 8 liquid oz. (240 ml)

Recovery Efficiency 78.7%, CV 5.6%
34.7% oocysts were not drained with the water

Method 1623, 10 liter volume 3 gallon volume = 11.3 liters

Filtration*^, Continuous Centrifugation, Flow Centrifugation, **Container Elution^**

Elution*, Dissolution^, Concentration, **IM Separation*^**, Purification, Flow Cytometry

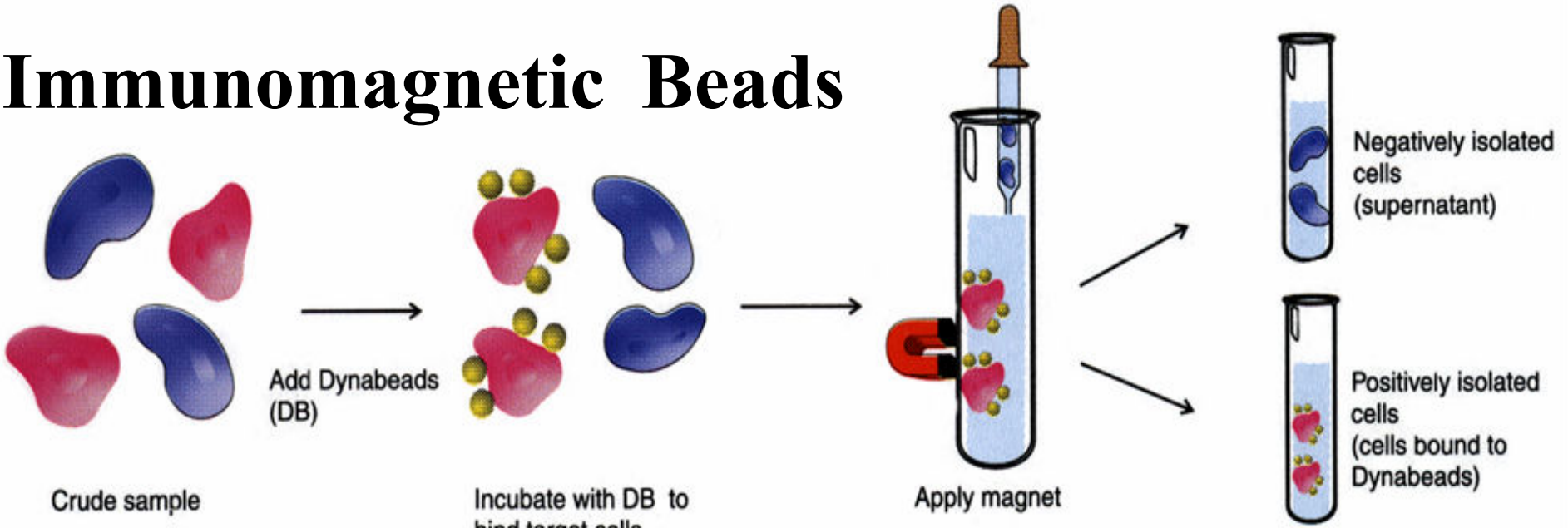
Staining*^, Examination*^, Enumeration*^

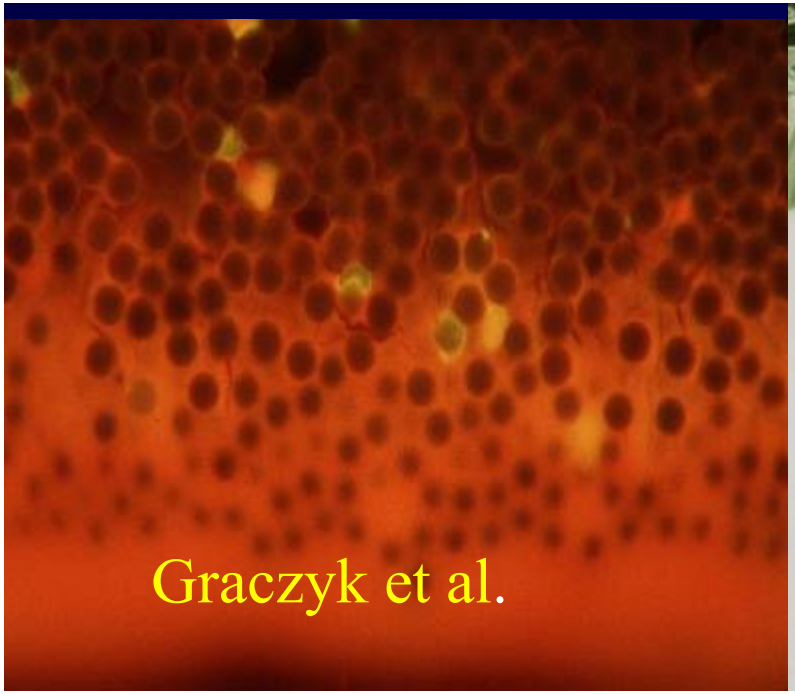
Water Decanted; Sediments Centrifuged and Purified by $CsCl_2$ Gradient Centrifugation

CAM-Filter Dissolution Method

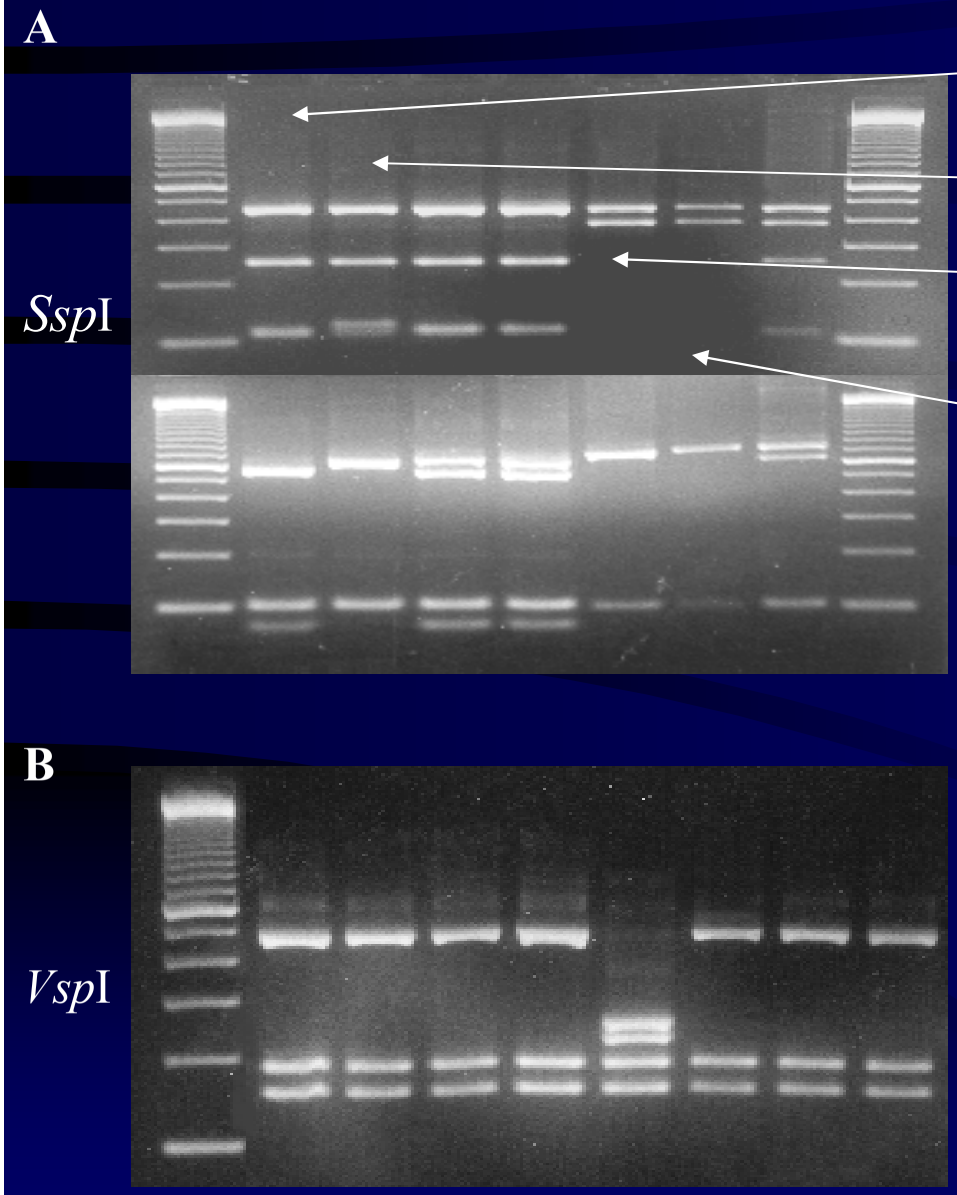
In vitro excystation, mouse bioassay, Fluorescent *In Situ* Hybridization (FISH)

Immunomagnetic Beads





Genotyping of *Cryptosporidium*; PCR, RFLP



C. hominis

C. parvum

C. andersoni

C. muris

Sewage; Wastewater discharges

vs.

Agricultural runoff

RECOVERY OF THE **NON–INFECTIOUS** ISOLATES OF *CRYPTOSPORIDIUM* BY POSITIVE REACTION WITH COMMERCIAL TEST KITS

Test Kit	Range of prevalence (%)
HYDROFLUOR	27 - 35
MERIFLUOR	27 - 35
PROSPECT	9 - 12

Graczyk et al. 1996, *Am J Trop Med Hyg* 54: 274-279

FISH Oligonucleotide Probes

- 5’ CGT TTA TCC ATG TAA GTA AAG 3’ CRY-1
- CGG CGG GGG GCC AAC TAC GIAR-4
- CGG GGC TGC CGC GGC GCG GIAR-6
- GTTC TCCTG CCCGT TTCAG INT-1
- ACTC ACAGG CATCC TTCAG CUN-1
- ACTC TCACA CTCAC TTCAG HEL-1

Fluorochromes: HEX Tet 6-Fam

FITC-conjugated mAb Combo *Cryptosporidium*/*Giardia*

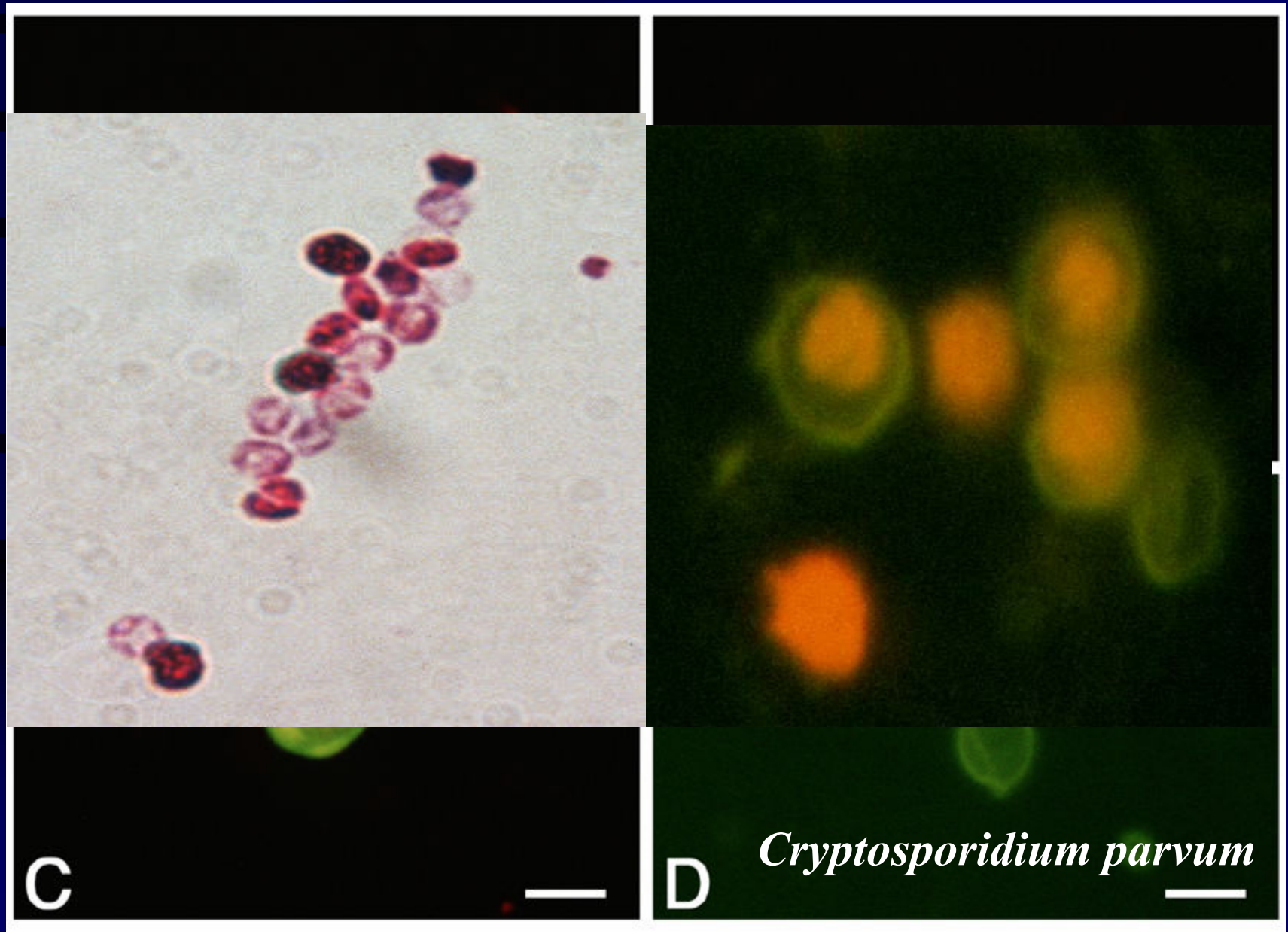
FISH Oligonucleotide Probes

- 5’ CGT TTA TCC ATG TAA GTA AAG 3’ CRY-1
- CGG CGG GGG GCC AAC TAC GIAR-4
- CGG GGC TGC CGC GGC GCG GIAR-6
- GTTC TCCTG CCCGT TTCAG INT-1
- ACTC ACAGG CATCC TTCAG CUN-1
- ACTC TCACA CTCAC TTCAG HEL-1

Fluorochromes: HEX Tet 6-Fam

FITC-conjugated mAb Combo *Cryptosporidium*/*Giardia*

Fluorescent I*n Situ* Hybridization (FISH)

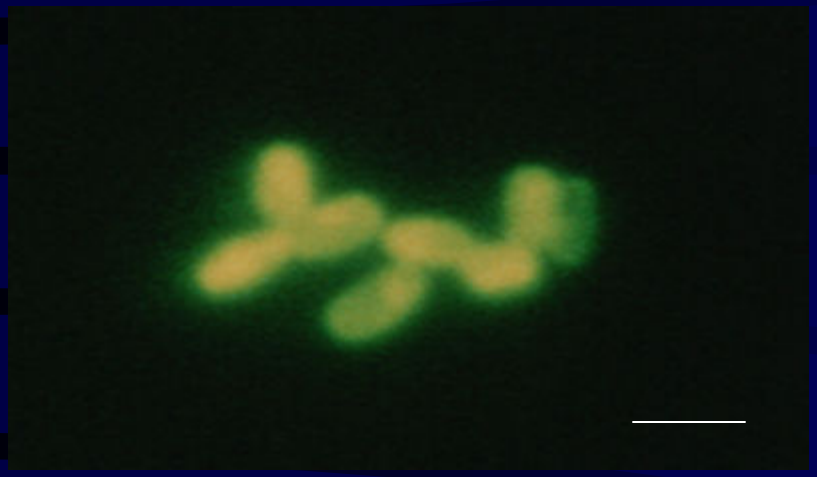


Correlation of FISH with Viability or Infectivity

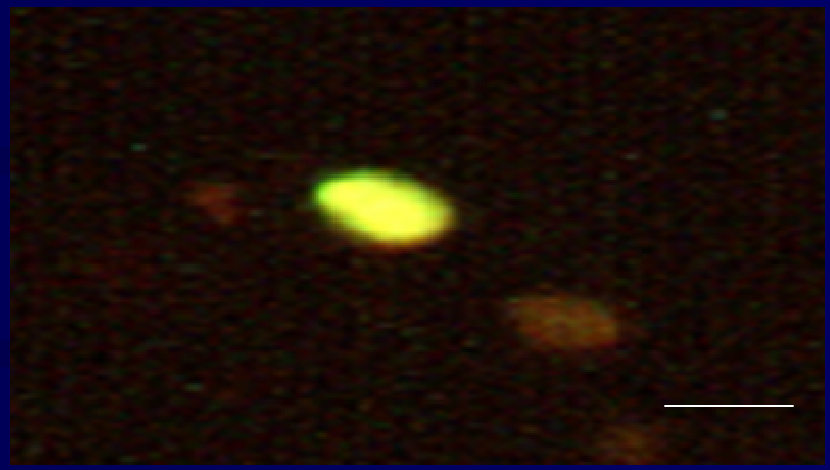
- Vessey et al. 1995. Protozoan Parasites and Water, Royal Soc Chem 133-138.
- Vesset et al. 1998. *J Appl Microbiol 85:* 429-440.
- Deere et al. 1998. *J Appl Microbiol* 85: 807-818.
- Jenkins et al. 2003. Parasitol Res 89: 1-5.
- Dorsch and Veal, 2001. *J Appl Microbiol* 90: 836-842.

Figure 2. Human-infective microsporidia identified by Fluorescent in Situ Hybridization. Scale bar 2 um

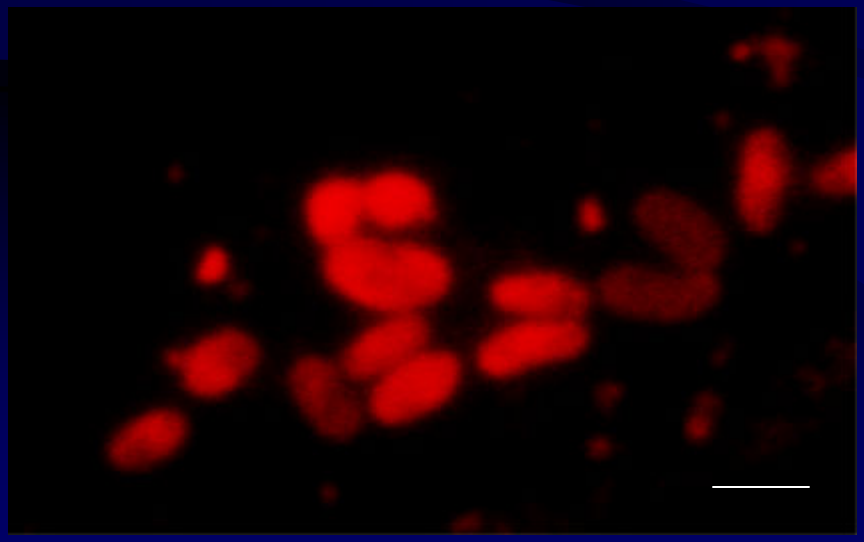
E. hellem



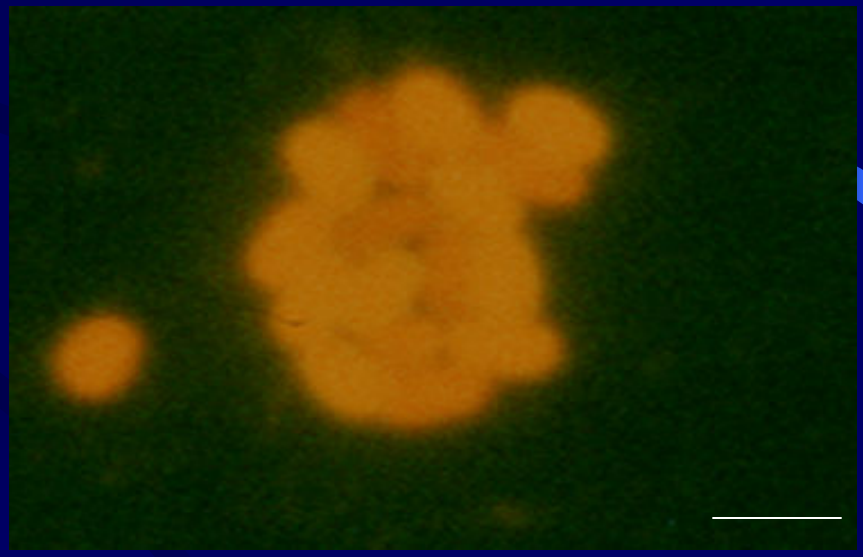
E. bieneusi



E. intestinalis



E. cuniculi



Procedure

1. Permeabilize in Acetone*
2. Wash w/ Hybridization buffer
3. Hybridize with probe for 1 hr at 57ºC
4. Wash w/H_2O
5. Make slides
6. Examine with an epifluorescent microscope or flow cytometry

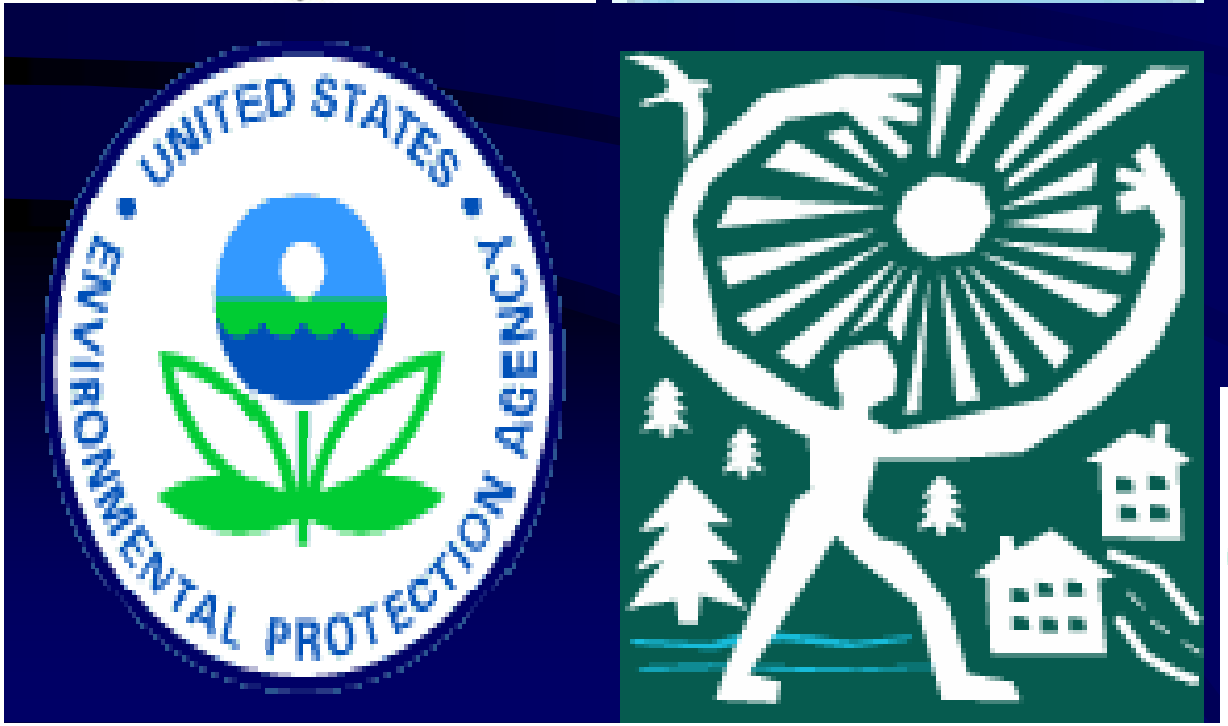
*** CAM-Filter Dissolution Method**

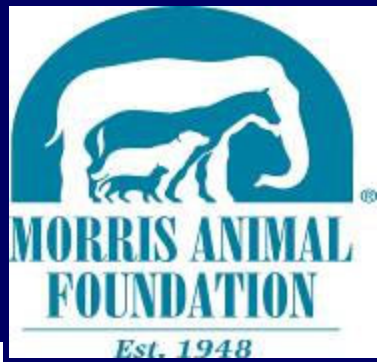
Combined FISH and IFA

- Sensitive and species-specific equivalent to PCR
- Determines viability of pathogen transmissive stages
- Applicable to environmental samples (lack of inhibition)
- Allows simultaneous detection of various pathogens in a sample (multiplex)
- Provides information on pathogen morphology
- Extraordinarily high resolution (single organism)
- Easy to use; cost, labor, and time effective


THE JOHNS HOPKINS
SCHOOL OF HYGIENE AND PUBLIC HEALTH
PER SCIENTIAM AD SALUTEM PUBLICAM
1916


MDE


UNITED STATES
ENVIRONMENTAL PROTECTION AGENCY


MORRIS ANIMAL
FOUNDATION
Est. 1948


Sea Grant
Mid-Atlantic

tgraczyk@jhsph.edu