ISOLATION AND CONCENTRATION OF *CRYPTOSPORIDIUM* FROM WATER

by

Andrew Charles Cifrino

A Thesis Submitted to the Faculty of the

DEPARTMENT OF NUTRITION AND FOOD SCIENCE

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN FOOD SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 8 6

STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Andrew Charles Gifkins

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

James W. Berry — December 8, 1986

James W. Berry
Professor of Nutrition and Food Science

Date

ACKNOWLEDGMENTS

I acknowledge the contribution of Cora Estabrook Musial. Musial developed the original method for the detection of Cryptosporidium oocysts which served as the basis for this research.

TABLE OF CONTENTS

LIST OF TABLES

Page

Table

ABSTRACT

A method was developed for the concentration and detection of Cryptosporidium oocysts in water. Oocysts were concentrated from 100 gallon volumes of tapwater using spun polypropylene cartridge filters. The filter was backflushed and washed with 0.1% Tween 80 in deionized water. Centrifugation of the eluate resulted in a suspension containing both oocysts and sediment. Density gradient centrifugation was used to remove the sediment. Membrane filtration of the final suspension was followed by labelling the oocysts with a fluorescein linked monoclonal antibody. Enumeration of the oocysts was accomplished by fluorescence microscopic observation of the membrane filter.

The recovery efficiency using this technique averaged 51%. Furthermore, the detection of as few as one oocyst per gallon of water was possible. This method can now be used for the investigation of waterborne outbreaks and to study the occurrence of Cryptosporidium in water.

CHAPTER 1

INTRODUCTION

Today 40% of acute cases of diarrhea are undiagnosed (Dupont, 1985). Between 1972 and 1981 Giardia Lamblia, a protozoan parasite, was shown to account for 15% of the waterborne outbreaks and 26% of the waterborne disease for the period (Craun, 1984). An outbreak of waterborne disease is a collection of one or more incidents of waterborne disease. Cryptosporidium is another protozoan parasite that shares many similarities with Giardia and is now emerging as a pathogen.

Cryptosporidium is a coccidian parasite, and so far only one species is recognized. The organism was first recognized by Tyzzer in 1907. In the 1970's sporadic cases of cryptosporidiosis in calves were identified. In 1976 the first human case was reported, and 58 cases, mostly in AIDS patients, had been reported by 1983 (Angus, 1983; Nime et al., 1976; Navine and Juranek, 1984). During the period from 1983 to 1985 various studies found 1-9% of diarrheal stools to be positive for Cryptosporidium (Montessori and Bischoff, 1985; Mata et al., 1984; Tzipori et al., 1983; Jokipii et al., 1983; Ratnam et al., 1985). A 1985 article in the

New England Journal of Medicine sites Cryptosporidium as one of the causes of diarrheal outbreaks at day-care centers (Dupont, 1985).

Cryptosporidium appears to be ubiquitous and to have little host specificity. It is found in birds, reptiles, mammals and fish (Current et al., 1983; Reese et al., 1982; Tzipori, 1983; Tzipori et al., 1980). It has also been found in travelers from Europe, school children in Costa Rica, and in peasants and cattle in Bangladesh (Wyllie, 1984; Mata et al., 1984; Shahid et al., 1985). Most outbreaks in this country have occurred among persons handling animals, but city dwellers in Australia have also been found to be infected by the organism (Current et al., 1983; White and Picklo, 1983).

Cryptosporidium is especially common in calves, and it inflicts great economic losses in the cattle and poultry industry. Anderson found 40% of the fecal samples from Idaho dairy calves to be positive for Cryptosporidium (Anderson and Hall, 1982). Because Cryptosporidiosis has been circumstantially linked with raw milk, Anderson studied milk pasteurization and its effect on Cryptosporidium. The organism was found to be extremely heat sensitive. With expanded use of recycled water for irrigation the possibility of transmission by contaminated vegetables should not be overlooked (Ma et al., 1985).

The immunological status of the individual is the major determinant of the course of cryptosporidiosis. In the immuno-competent host the illness typically lasts for one to ten days and results in diarrhea without blood. The disease is self-limiting and may be asymptomatic. In the immuno-compromised host the disease causes severe persistent diarrhea with serious fluid loss. T-cells are required for recovery (Current et al., 1983; Moon, 1986). In the past, diagnosis was accomplished with intestinal biopsies which were examined by light or electron microscopy. Acid-fast stains have been used for the detection of Cryptosporidium (Garcia et al., 1984; Ma and Soave, 1983). Today the oocyst state of Cryptosporidium is detected with a direct immunofluorescence technique (Sterling and Arrowood, n.d.). At this time there is no effective drug treatment for Cryptosporidiosis (Moon, 1986).

Infection begins with ingestion of oocysts and liberation of sporozoites which infect epithelial cells of the gut. The sporozoites reside in an intracellular extracytoplasmic location. There is a general disordering of the epithelial cells with concomitant cellular exudate and smaller villus (Moon, 1986).

The loss of absorptive cells results in impaired carbohydrate absorption which in turn results in water loss by osmosis and diarrhea. Dr. Harley Moon of the

National Animal Disease Center measured decreased xylose absorption in infected calves (Moon, 1986). The infection moves down the GI tract as it keeps spreading to new epithelial tissue, while the organism multiplies asexually and develops through various life-stages. Eventually the cyst stage is reached. Because Cryptosporidium eventually reaches a sexual stage, this cyst is called an oocyst. These thick walled capsules or oocysts protect the parasite in the external environment (Noble and Noble, 1971). In transmission it is the oocyst that is passed by contaminated food or water and by the presence of oocysts on hands or clothing (Current, 1984). Waterborne transmission is likely because high numbers of oocysts are shed in feces and they are extremely resistant to chlorine (Campbell et al., 1982). Chlorine is the disinfectant commonly used in water treatment systems.

The aim of this project was to enhance the recovery efficiency of a method developed for the concentration and detection of Cryptosporidium in water.

CHAPTER 2

METHODS AND MATERIALS

In these experiments Cryptosporidium oocysts were isolated from tapwater and enumerated by microscopic observation. Preliminary experiments to determine the best method for isolation and detection of oocysts were performed using 20 liter volumes of dechlorinated tapwater. The results of these early studies are not appropriate for interpretation, because the experimental methods were constantly evolving. Trial and error and the principle that many small washings of the filter are preferable to a single large washing resulted in a simplification of the method developed by Musial (Musial, 1985). The method developed and described below was used to study the distribution of oocysts in the filter and the efficiency of 378 liter filtrations.

Preparation of the Inoculum

Oocysts used in these experiments were supplied by Dr. Charles R. Sterling, Department of Veterinary Science, University of Arizona, Tucson. These oocysts were supplied in 50 ml aliquots as a suspension in 2.5% potassium dichromate. These suspensions, contained in 50

ml screw-top polypropylene tubes, had titers of $10^6 - 10^7$ oocysts per ml. Polypropylene equipment was used throughout these experiments to reduce losses of oocysts due to adsorption.

For the 378 liter filtration experiments and the study of oocyst distribution in the filter, inocula were prepared by placing $10^2 - 10^7$ oocysts in 200 ml tapwater dechlorinated by addition of 2 drops of a 10% sodium thiosulfate solution. One hundred ml was used for enumeration, and the remaining 100 ml was seeded into 378 liters of dechlorinated tapwater for filtration. For the elution and flotation experiments inocula were prepared by adding $10^3 - 10^4$ oocysts to 100 ml dechlorinated tapwater. Fifty ml was reserved for enumeration, and the remaining 50 ml was inoculated in four 12 ml aliquots into four 378 liter volumes for a total of 1512 liters.

For the Sheather's flotation experiments approximately 10^4 oocysts were added to 20 ml of a solution made by mixing 40 ml of 1% Tween-80 with 60 ml of 1% sodium dodecyl sulfate (SDS) in a 50 ml plastic beaker. The solution was constantly stirred, and 1 ml aliquots were removed for layering onto the Sheather's solutions and the seed count. For the Sheather's flotation experiments with sediment, 4 ml of the inoculum, prepared as above, was mixed with a quantity of Tucson tapwater sediment about equal to four times that quantity found in

378 liters of Tucson tapwater. After vortexing the resultant suspension was divided into four aliquots and layered onto four Sheather's solutions.

Filtration

For all experiments except the study of oocyst distribution in the filter, the seeded tapwater was filtered through a 5-inch (Contamination Technology, Phoenix, AZ) or 10-inch spun fiber polypropylene cartridge filter (AMF/Cuno Division, Meriden, CT), nominal porosity of 1 μm, at 18.9 liters per minute. An electric pump (Jabsco Products, Costa Mesa, CA) with the flow control valve set fully open was used to maintain the above flow rate. For the study of oocyst distribution in the filter 20 liter volumes were filtered at 3.78 liters per min using a 20 liter pressure vessel (AMF/Cuno Division, Meriden, CT). Ten psi nitrogen gas was used to maintain this flow rate.

Filter Elution

To determine the distribution of oocysts in the filter, the filter (10-inch cartridge) was backflushed with 2000 ml deionized water (DI) with 1% Tween 80 at 10 PSI. The eluate was divided into six portions and the filter was divided into three parts. Each filter section, inside, middle and outside, was pulled apart by hand and washed twice with approximately 300 ml eluate per wash on

a mechanical shaker. The filter material was discarded and the eluate centrifuged twice to pellet back both oocysts and sediment. All centrifugations were performed at 1,200 x g (2,500 rpm) in an IEC Centra-7 bench top centrifuge with a 210 rotor for 10 min unless otherwise noted. In one trial a 50 ml portion of the backflushed eluate was reserved to enumerate the oocysts dislodged from the filter by backflushing alone. In this experiment a high inoculum, i.e. 10^6 oocysts/20 liters, was used. Because of the large number of oocysts, no purification step was required prior to enumeration of oocysts.

In the 378 liter filtration experiments the filter was backflushed with 2700 ml DI containing 3 ml Tween 80 at 10 psi N_2 gas. The filter material was cut from its core, pulled apart by hand, and washed three times with 900 ml eluate per wash on a mechanical shaker. The filter material was discarded and the eluate was centrifuged 3 times to pellet both the oocysts and sediment. Again, when a high inoculum, 10^3 oocysts/378 liters or greater, was used, sediment removal was not required for enumeration of the oocysts. In the low inoculum trials (trials 4-6, Table 6) sediment was removed by flotation on 3/5 strength Sheather's. Full strength Sheather's consisted of 500 g sucrose in 320 ml DI with 9.7 ml liquid phenol. Dilutions were made by adding DI. Also, all

equipment was soaked in 1% bleach for 30 min before each low inoculum trial.

Sediment Removal

For the low inoculum filtration trials and the attempted environmental assays, sediment removal was required. In selecting the appropriate flotation media zinc sulfate and potassium citrate were eliminated as candidates, because they were incompatible with other reagents. Flotation experiments were done using Sheather's, saturated saline, and Percoll solutions. For the saturated saline flotation the centrifugation was at 450 x g (1400 rpm) for 2 min. The Percoll gradient consisted of a 9:1:9 solution of Percoll:10X Alsevers: Alsevers. The procedure used for oocyst isolation on the Percoll gradient is given in the A appendix. In these experiments 1512 liters of dechlorinated tapwater (dechlorinated by addition of 40 ml of a 10% sodium thiosulfate solution per 378 liters) were filtered in 378 liter aliquots. In trials 1 and 2 each of the 378 liter aliquots was inoculated with approximately 800 oocysts and filtered. In trial 3 each 378 liter aliquot was inoculated with 8000 oocysts and filtered. The filter was processed as described above for the 378 liter filtrations to obtain a suspension of oocysts and sediment. This suspension was divided into four aliquots

and layered onto 1) full strength Sheather's, 2) full strength Sheather's after homogenization and sonication, 3) saturated saline after homogenization and sonication and 4) Percoll gradient solution after homogenization and sonication. Homogenization was performed in a VirTis '45' homogenizer (VisTis, Gardener, NY) on setting 20 for 2 min. Sonication was performed in a water bath sonicator (Ultrasonic Cleaning System E-module, Branson Cleaning Equipment Co., Shelton, CT) for 4 min.

A second set of flotation experiments was done to find the optimal concentration of Sheather's for oocyst recovery. The concentrations used were full strength, 4/5, 3/5, 2/5, 3/10, 1/5 and 1/10 full strength respectively. The specific gravity for each solution was measured using a set of hydrometers. Suspensions of oocysts both with and without sediment were prepared, divided into four aliquots, and layered onto four different strength Sheather's solutions. After centrifugation the oocysts were recovered by collecting the supernatant, diluting it by a factor of 5 or more with DI and centrifuging again to pellet the oocysts. The pelleted oocysts were then enumerated.

Oocyst Detection

To enumerate oocysts 1 ml volumes of the oocyst suspension were filtered through 13 mm diameter

polycarbonate membrane filters (Nuclepore Corporation, Pleasanton, CA) with a 1.2 μm pore size. The filter was rinsed with 1% Tween 80 in phosphate buffered saline (PBS), and the antibody solution was added directly to the filter. To make the antibody solution 0.2 ml stock antibody, obtained from Dr. Sterling, was diluted 1:50 in 9.8 ml PBS and 0.1% sodium azide. Both direct and indirect monoclonal antibody systems were used. With the indirect system the fluorescein isothiocyanate is conjugated to an antibody which binds to another antibody which has previously been incubated with the oocysts. That is, it is a two-step process and so, two incubation periods (20 min each) were required. With the direct system the fluorescein isothiocyanate is conjugated to an antibody which binds directly to the oocyst wall. The exact site of binding is not known, and both antibody systems are not perfectly specific for the oocyst wall. The direct antibody system is known to cross react with yeasts and pollen, and the indirect antibody system cross reacts with sediment (Arrowood, 1986). The indirect antibody system was used only for the environmental assays. After incubation with the antibody solution the filter was rinsed twice with Tween 80-PBS and mounted on a slide with a glycerol-PBS solution adjusted to pH 8 for fluorescence microscopic (Nikon Inc., Garden City, NY) observation at 400X. The number of oocysts in the sample

was determined by the formula: (oocysts counted/ml) (dilution factor) (volume of final concentrate in ml) = number oocysts. For example, if 30 oocysts were counted in 1 ml of the final concentrate (no dilutions were made so the dilution factor is 1×10^{o} or unity) and the final concentrate volume was 5 ml, then the total number of oocysts collected is determined as follows:

$$(30 \text{ oocysts counted/1 ml}) (1 \times 10^{o})$$

$$(5 \text{ ml}) = 150 \text{ oocyts}$$

Note that the units of volume cancel, and the number obtained is the total number of oocysts collected in the final concentrate. This number was divided by the initial inoculum size, determined in the same manner, and multiplied by 100 for percent recovery. For the elution and flotation experiments percent recovery was determined as above for each flotation method. Total efficiency was then calculated by adding the numbers obtained in each flotation, dividing by the initial inoculum size and multiplying by 100.

Environmental Assays

Three environmental samples were assayed. One well and one storage tank sample were obtained from a local private water company. The third sample was secondary effluent from a local waste-water treatment plant. In the latter case two samples were inoculated

with 10^7 oocysts/100 gallons in addition to the environmental assay. Uninoculated negative control studies were performed at various steps and times to make certain that contaminated equipment did not contribute to the results. Autoclaving, dry heat and washing with bleach and water were all tried as a means of rendering the equipment free of oocysts.

CHAPTER 3

RESULTS

Distribution of Oocysts in the Filter

The distribution of oocysts in the filter (Table 1) shows that during filtration oocysts did penetrate to the inner region of the filter. Trial #3 suggests that backflushing successfully dislodges oocysts from the filter.

Efficiency of Elution and Flotation

The efficiency of elution and flotation was evaluated for four different flotation methods (Table 2). Sheather's flotation both with and without sonication and homogenization yielded better recoveries than saturated saline or Percoll flotations. Sonication and homogenization do not appear to enhance the recovery of oocysts from suspensions of tap water sediment for the Sheather's flotations.

Recovery of Oocysts by Sheather's Flotation

The recovery of oocysts from suspensions both with and without sediment was also examined (Tables 3-A, 3-B and 3-C). Full strength Sheather's and dilutions of the full strength Sheather's were used in these flotations.

Table 1. Distribution of Oocysts in Filter

Trial	Inoculum oocyst /20L	Total Number Recovered ($\times 10^6$) Filter Section: Inside	Middle	Outside	Back Flush	Efficiency
1[a]	9.4	0.90 (20%)[b]	1.5 (34%)	2.0 (45%)	Not Back Flushed	47%
2	16.0	3.90 (26%)	5.0 (33%)	6.1 (41%)	Not Back Flushed	94%
3	5.0	0.42 (9.4%)	1.47 (33%)	0.97 (22%)	1.6 (36%)	89%

[a] All counts for trial 1 were made in duplicate and averaged

[b] Numbers in parentheses are percent recoveries

Table 2. Efficiency of Elution and Flotation

Trial	Inoculum Oocysts/ 1512 Ltr	Total Number Recovered: Sheather's w Sonication & Homogenization	Sheather's w/o Sonication & Homogenization	Saturated Saline	Percoll	Total Efficiency
1	3499	1129	760	520	496	85%
		(39)[a]	(26)	(18)	(17)	
2	3080	320	440	104	92	31%
		(33)	(46)	(11)	(9.6)	
3[b]	30800	1280	1560	272	432	12%
		(36)	(44)	(7.7)	(12)	
Avg.		36%	39%	12%	13%	43%

[a] Numbers in parentheses are percent recoveries

[b] Total efficiency lowered because pump impeller fragmented and thereby contaminated preparation

Table 3-A. Recovery of Oocysts from Suspensions Without Sediment Using Sheather's Flotation.

	Percent Recoveries						
	Sheather's Solution (sp. gr.)[a]						
Trial	Full Strength (1.29)	4/5 (1.24)	3/5 (1.17)	2/5 (1.11)	3/10 (1.08)	1/5 (1.06)	1/10 (ND)
1	44	42	63	38			
2			68	84	73	40	
3				75	68	38	37
4	81	89	79	88			
Avg.	63	66	70	71	71	39	37

[a] Specific gravity

Table 3-B. Recovery of Oocysts from Suspensions with Tapwater Sediment Using Sheather's Flotation

	Percent Recoveries					
	Sheather's Solution (sp. gr.)[a]					
Trial	Full Strength (1.29)	4/5 (1.24)	3/5 (1.17)	2/5 (1.11)	3/10 (1.08)	1/5 (1.06)	1/10 (ND)
1		53	92	69	70		
2				66	57	12	7.3
3		112	76	72	64		
4			59	45	22	2.8	
Avg.			76	63	53	7.4	7.3

[a] Specific gravity

Table 3-C. Recovery of Oocysts from Suspensions Both With and Without Sediment Using Sheather's Flotation

	Combined Percent Recoveries						
	Sheather's Solution (sp. gr.)[a]						
	Full Strength (1.29)	4/5 (1.24)	3/5 (1.17)	2/5 (1.11)	3/10 (1.08)	1/5 (1.06)	1/10 (ND)
Avg.	63	74	73	67	59	23	22

[a] Specific gravity

Full strength, 4/5, 3/5, 2/5, 3/10, 1/5 and 1/10 dilutions were tested, and the recoveries averaged 63, 74, 73, 67, 59, 23 and 22% respectively. There is a statistically significant difference ($p=0.05$) between the recoveries measured using the 1/5 and 1/10 strength Sheather's and the recoveries measured using the full strength 4/5, 3/5, 2/5 and 3/10 strength Sheather's (Table 3-C).

Recovery of Oocysts in 378 Liters

The efficiency of oocyst detection in tap water using 5 inch or 10 inch cartridge filters was determined (Tables 4 and 5 respectively). Recovery averaged 51% for all trials with a range of 25-81%. The average recovery was 34% in the low inoculum studies and 58% in the high inoculum studies.

Preliminary Environmental Studies

Preliminary environmental studies using these methods were undertaken. The results of samples taken from a local water company are inconclusive. These samples were heavily laden with sediment and could not be adequately purified. In two inoculated studies at a local water treatment plant oocyst recovery from secondary effluent averaged 7%. The single uninoculated secondary effluent sample taken yielded 170 oocysts per gallon. Samples taken at the local water treatment plant were assayed using both a direct and an indirect monoclonal

Table 4. Recovery of Oocysts from Tapwater with 5 Inch Cartridge Filter

Trial	Total Number of Oocysts ($\times 10^3$) Inoculated Tapwater	Concentrate	% Recovered
1	5000	2800	56
2[a]	1.6	1.2	75
3	1.8	1.0	56
4	1.1	0.48	44
		Average	58 ± 11

[a] Both counts for trial 2 were made in duplicate and averaged

Table 5. Recovery of Oocysts from Tapwater With 10 Inch Cartridge Filter

	Total Number of Oocysts ($\times 10^3$)		
Trial	Inoculated Tapwater	Concentrate	% Recovered
1	2500	630	25
2[a]	2600	2100	81
3	2300	1600	70
4[b]	0.131	0.055	42
5	0.170	0.053	31
6	0.177	0.051	29
		Average	46 ± 24

[a] Both counts for trial 2 and 3 were made in duplicate and averaged

[b] For trials 4-6 entire preparation was examined and counted

antibody system. The direct antibody cross reacted with yeasts and pollen, therefore false positives were possible. The indirect antibody cross reacted with sediment resulting in background fluorescence which rendered the detection of oocysts difficult at best.

Negative Control Experiments

In the negative control experiment performed between trials 5 and 6 for the 10 inch filter after all equipment had been washed with bleach and water, no oocysts were seen. However, unidentified fluorescing objects, probably yeast or pollen, were seen with both the direct and indirect antibody (Arrowood, 1986). In a second negative control experiment, performed after the first inoculated secondary effluent study, in which the equipment had not been thoroughly soaked in bleach and water, 12 oocysts were recovered from the equipment. In other negative control experiments 1-20 oocysts were recovered depending on the extent to which the equipment had been cleaned with bleach and water. Autoclaving and dry heat (3 hrs at 120 C) were not effective means of cleaning the equipment.

CHAPTER 4

DISCUSSION

Factors Influencing the Recovery of Oocysts from Water

There are several factors that may influence the efficiency of this method. These include: 1) flow rate, 2) filter characteristics, 3) the purification method and 4) the enumeration process.

While oocysts penetrated deeply into the filter during the 20 liter filtrations at 3.78 liters/min (Table 2), the majority were concentrated in the outer region of the filter. The 1512 liter filtration experiments in which inoculated tap water was filtered in 378 liter aliquots at 18.9 liters/min (Table 2) demonstrate the ability of the filter to trap and retain oocysts even at the increased flow rate. Musial reported "greatly reduced" recoveries at the higher flow rate and reasoned that oocysts were deeply embedded into the filter at the higher flow rate and therefore more difficult to elute from the filter (Musial, 1985). This reasoning appears to be correct, because the method has now been modified to include multiple washings of the filter, and a reduction in recovery is no longer seen at the higher flow rate.

The 378 liter filtration experiments were performed with both 5 inch and 10 inch cartridge filters, and the construction of each size is different. Even though both filters have the same nominal porosity of 1 um, the 10 inch filter collected more sediment than the 5 inch filter during tap water filtrations. Because tap water sediment was easily removed by density centrifugation and because both filter types are equally effective at trapping the coarser sediments found in environmental water, this consideration is not too important. Slightly more oocysts were recovered in the 378 liter filtration experiments using the 5 inch filter than with the 10 inch filter (Tables 4 and 5), but more low inoculation trials were performed with the 10 inch filter. Because the average recovery in the low inoculum trials was low relative to the high inoculum trials, the average recovery for the 10 inch filter might be low, because more low inoculum trials were performed using the 10 inch filter. The differences in efficiency, if any, between the two filters needs to be evaluated. The 5 inch filter is recommended, because its construction makes easier the dismantling required for elution.

The lower percent recovery in the low inoculum trials is best explained by losses of oocysts due to adsorption to the equipment. The presence of 1-20 oocysts

in uninoculated negative control studies in which the equipment had not been thoroughly cleaned demonstrates that percent recoveries can be inflated by contamination. However, the low inoculation trials in Table 5 (trials 4-6) were performed after all equipment had been thoroughly cleaned with bleach and water. The negative control studies show this cleaning procedure is effective.

Oocysts tend to stick to one another as well as to the equipment. The large variance in the data is probably the result of the tendency for oocysts to clump together. These clumps, which looked like clumps of grapes, made counting difficult and skewed results. The presence or absence of just one clump could drastically alter the count. Musial found a suspension of oocysts containing 0.1% concentration of Tween-80 gave significantly higher counts than a similar suspension of oocysts made from the same stock suspension without Tween-80 (Musial, 1985). A 1% concentration of Tween-80 was effective at breaking up clumps of oocysts. Clumps were not observed in the low inoculum trials.

In four trials using an inoculum of 10^2 oocysts Musial recovered an average of 15% from 378 liter filtrations. In these experiments with the same inoculum size the recovery for three trials using a modification of the Musial method averaged 34%. An even more dramatic

increase in percent recovery was observed for the trials with an inoculum size of 10^3 oocysts per 378 liters. The modifications most likely to be responsible for this improvement are multiple washings of the filter and the modified procedure for enumeration which is described below. There were no similar improvements in percent recovery for filtrations of inoculated secondary effluent. One explanation for this lack of improvement is that the filtrations were made in May and June when the secondary effluent is heavily laden with algae. Many alga have a density close to that of the oocysts and are difficult to remove by purification.

It is in the concentration and purification steps where critical losses are incurred. It is essential to remove sediment and partially purify the sample in order to detect the oocysts. Purification by Sheather's flotation is a critical step where recoveries may be increased or decreased. The 3/10 strength Sheather's solution with a specific gravity of 1.09 provided the minimum density required to retain oocysts in the supernatant. However, density is just one consideration in determining the suitability of a particular flotation medium. Another consideration is the presence of salts. Even though saturated saline had a specific gravity of 1.12, slightly greater than the value for 3/5 strength

Sheather's, the recoveries from saturated saline flotations were low. The low recoveries for saturated saline and Percoll flotations (Table 2) are best explained by the formation of salt bridges between oocysts and sediment particles (Bitton, 1980).

The 3/10 strength Sheather's provided the minimum density required for oocyst flotation, but a denser Sheather's solution is recommended to prevent sediment from descending very rapidly during centrifugation with the result that oocysts descend with it. Rapidly descending sediment is likely to entrap oocysts and thereby pellet both oocysts and sediment. It is also likely that the vortices created by rapidly descending sediment resulted in a downward migration of oocysts.

A two step density centrifugation process is recommended for more efficient purification. In the first step the sample would be layered onto full strength Sheather's solution, and subsequent centrifugation would remove only the coarser and more dense sediment. Another flotation on a less dense Sheather's solution could then be used for removal of the finer, less dense sediment. This two step process would prevent the loss of oocysts due to entrapment in a rapidly descending pellet of sediment. Another recommendation is the use of a screen

mesh to act as a prefilter for the intake hose during filtration.

The role of sonication and homogenization as a means of separating oocysts from sediment needs to be evaluated for many sediment types. For tap water sediment sonication and homogenization do not appear to enhance recoveries (Table 2). However, Musial found a combination of Tween-80, SDS, and sonication was required for recovery of oocysts from suspensions of tap water sediment. The use of SDS and Tween-80 is recommended by Musial and was incorporated into all suspensions by vortexing before the sample was layered onto the Sheather's solution.

Membrane filtration of the final concentrate followed by staining the oocysts directly on the filter enables the examination of a larger sample volume than possible with a hemacytometer. It is also more convenient than heat fixing the oocysts to poly-L-lysine coated slides as was done in the past (Musial, 1985). For the 378 liter tapwater filtration experiments four membrane filters were sufficient to examine the entire sample. The lack of perfect specificity in the antibody system requires some familiarization with the oocysts before accurate counts can be made. This difficulty in making accurate identification of the etiological agent involved in outbreaks of waterborne disease contributes to the fact

that in 50% of all outbreaks since 1946 no causative agent has been identified (Craun, 1984).

APPENDIX A

PROCEDURE FOR CRYPTO OOCYST ISOLATION ON PERCOLL GRADIENT

PROCEDURE FOR CRYPTO OOCYST ISOLATION ON PERCOLL GRADIENT

Reagents or Solutions Needed:

10x Alsever's
1x Alsever's
Percoll
PBS
2.5% dichromate

---

1. Place 8 high-speed polycarbonate test tubes in a holder.

2. Take out 2 Secondary Sheather's tubes of oocysts. Spin for 15 minutes at 3000 RPM. Vacuum off supernatant and resuspend each pellet with 4 mls of saline or PBS.

3. For the Percoll gradient use a 9:1:9 solution of Percoll: 10X Alsever's: Alsever's. For a full run of eight tubes use:
   34 ml Percoll
   4 ml 10x Alsever's
   34 ml 1x Alsever's

4. Mix the gradient solution thoroughly and dispense 9 ml into each polycarbonate tube.

5. If not to be used immediately, keep gradient tubes refrigerated!

6. Four Percoll tubes will be used for each tube of Secondary Sheather's cleaned oocysts. Be careful to label which Percoll tubes go with which Sheather's tube.

7. Carefully overlay each Percoll gradient with 1 ml of the Sheather's cleaned oocyst material.

8. Centrifuge the tubes at 13,000 RPM for 30 minutes in SS-34 angle rotor in Sorvall high-speed centrifuge.

9. Vacuum off heavy or orange top band into vacuum jar.

10. Collect oocysts in solution down to about 1 ml, depending upon the density of oocysts present.

11. Dispense oocyst solution into labeled 50 ml centrifuge tubes.

12. Resuspend each solution to 8 ml in PBS or saline and centrifuge at 3,000-4,000 RPM for 25 minutes.

13. Vacuum off solution and resuspend pellet. Wash as in step 12.

14. Resuspend final pellet to 20 ml with 2.5% dichromate.

15. Make sure all pellets from one sample of Secondary Sheather's oocysts run on Percoll are combined into one final 50 ml blue-capped tube and labeled with name of calf and date tube was run.

16. Refrigerate oocyst sample and allow to sit overnight.

17. Next morning: Make a 1:5 or 1:20 dilution of oocyst sample to PBS and count oocyst number on hemacytometer. Label tube with oocyst count per ml.

18. Record all data in record book.

19. Re-refrigerate all tubes.

20. Wash all used tubes in Chlorox. Wash down workspace in Chlorox.

REFERENCES

Anderson, B. C., and R. F. Hall. 1982. Cryptosporidial infection in Idaho dairy calves. J. Am. Vet. Assoc. 181:484-485.

Angus, K. W. 1983. Cryptosporidiosis in man, domestic animals and birds: A review. J. Roy. Soc. Med. 76:62-69, 1983.

Arrowood, M. J. 1986. Graduate student, Department of Microbiology and Immunology, The University of Arizona, Tucson. Personal communication.

Bitton, G. 1980. Introduction to environmental virology. New York: John Wiley and Sons.

Campbell, I., S. Tzipori, G. Hutchison, and K. W. Angus. 1982. The effect of disinfectants on survival of Cryptosporidium oocysts. Vet. Rec. 222:414-415.

Craun, G. F. 1984. Waterborne outbreaks of giardiasis: current status. p. 243-261. In S. L. Erlandsen and E. A. Meyer (ed.), Giardia and giardiasis. New York: Plenum Press.

Current, W. L. 1984. Cryptosporidium and cryptosporidiosis. In M. S. Gottleib and J. D. Groopman (ed.), Acquired immune deficiency syndrome. New York: Alan R. Liss, Inc., p. 355-373.

Current, W. L., N. C. Reese, J. V. Ernst, W. S. Bailey, M. B. Heyman, and W. M. Weinstein. 1983. Human cryptosporidiosis in immunocompetent and immunodeficient persons: studies of an outbreak and experimental transmission. N. Engl. J. Med. 308:1252-1257.

Dupont, H. L. 1985. Cryptosporidiosis and the healthy host. N. Engl. J. Med. 312:1319-1320.

Garcia, L. S., D. A. Bruckner, T. C. Brewer, and R. Y. Shimizu. 1984. Techniques for the recovery and identification of Cryptosporidium oocysts from stool specimens. J. Clin. Microbiol. 18:185-190.

Jokipii., L., S. Pohjola, and A. M. M. Jokipii. 1983. Cryptosporidium: A frequent finding in patients with gastrointestinal symptoms. Lancet 1152:358-361.

Ma, P., D. L. Kaufman, C. G. Helmick, A. J. D'Souza, and R. R. Navin. 1985. Cryptosporidiosis in tourists returning form the Caribbean. N. Engl. J. Med. 312:647-648.

Ma, P., and R. Soave. 1983. Three step stool examination for cryptosporidiosis in ten homosexual men with protracted watery diarrhea. J. Infect. Dis. 147:824-828.

Mata, L., H. Bolanos, D. Pizzaro, and M. Vines. 1984. Cryptosporidiosis in children from some highland Costa Rican rural and urban areas. Am. J. Trop. Med. Hyg. 33:24-29.

Montessori, G. A., and L. Bischoff. 1985. Cryptosporidiosis: A cause of summer diarrhea in children. Can. Med. Assoc. J. 132:1285.

Moon, H. W. 1986. National Animal Disease Center, Ames, Iowa. Personal communication.

Musial, C. E. 1985. Development of a method for the detection of Cryptosporidium in water. Dissertation at the University of Arizona, Department of Microbiology and Immunology, Tucson, Arizona.

Navin, T. R., and D. D. Juranek. 1984. Cryptosporidiosis: Clinical epidemiologic and parasitologic Review. Rev. Infect. Dis. 6(B):313-327.

Nime, F. A., J. D. Burek, D. L. Page, M. A. Holscher, and J. H. Yardley. 1976. Acute enterocolitis in a human being infected with the protozoan Cryptosporidium. Gastroenterology 70:592-598.

Noble, E. R., and G. A. Noble. 1971. Parasitology. Philadelphia: Lea and Febiger.

Ratnam, S., J. Pakkock, E. McDonald, D. Whitty, M. Jong, and R. Cooper. 1985. Occurrence of Cryptosporidium oocysts in fecal samples submitted for routine microbiological examination. J. Clin. Micro. 22:402-404.

Reese, N. C., W. L. Current, J. V. Ernst, and W. S. Bailey. 1982. Cryptosporidiosis of man and calf: a case report and results of experimental infections in mice and rats. Am. J. Trop. Med. Hyg. 31:226-229.

Shahid, N., A. S. M. H. Rahman, B. C. Anderson, L. J. Mata, and S. C. Sanyal. 1985. Cryptosporidiosis in Bangladesh. Br. Med. J. 290:114-115.

Sterling, C. R., and M. J. Arrowood. 1987. Detection of Cryptosporidium sp. infections using a direct immunofluorescent assay. Pediatr. Infect. Dis. In press.

Tzipori,S., M. Smith, C. Birch, G. Barnes, and R. Bishop. 1983. Cryptosporidiosis in hospital patients with gastroenteritis. Am. J. Trop. Med. Hyg. 32:93911-934.

Tzipori, S. 1983. Cryptosporidiosis in animals and humans. Microbiol. Rev. 47:84-96.

Tzipori, S., K. W. Angus, I. Campbell, and E. W. Gray. 1980. Cryptosporidium: evidence for a single-species genus. Infect. Immun. 30:884-886.

White, W. L., and J. Picklo. 1983. Human Cryptosporidiosis. N. Engl. J. Med. 309:1325.

Wyllie, A. S. 1984. Cryptosporidiosis. Br. Med. J. 289:1383-1384.