FIELD METHODOLOGY DESCRIPTION AND CORRELATIONAL ANALYSIS OF WELL WATER CONTAMINATION AND DIAHRREAL DISEASE IN CHILDREN OF TRANSYLVANIA REGION, ROMANIA

Rick J. Bauer [a], Mariana Vlad [b], Danielle Moga[b], Auelian Sinca [b], Ileana Mirestean [b], and Catherine L. Zeman* [c]

[a] Indiana Clean Manufacturing Technology & Safe Materials Institute, School of Civil Engineering, Purdue University 2655 Yeager Rd. Suite 103, West Lafayette, IN 47906-1337, USA
[b] Institute of Public Health, Cluj-Napoca 6, Louis Pasteur St. 3400 Cluj-Napoca Romania
* [c] Corresponding author, Health Division, School of HPELS, University of Northern Iowa, Cedar Falls, Iowa 50614, USA. Telephone: 319-273-7090, FAX: 319-273-3689, Email: catherine.zeman@uni.edu

Abstract

Sixty-nine wells in the Transylvania region of Romania were analyzed for the presence and concentration of *Giardia* cysts, *Cryptosporidium* oocysts, and E. coli. These wells have been under study by the corresponding author since 1997 and a good deal of data concerning well characteristics and location was supplemented by concurrently interviewing well owners about their children's health history to determine if a correlation exists between the various water quality risk factors, local environment and environmental practices and the frequency and average severity of diarrheal disease in the study group. This necessitated the development of a collection, elution, concentration, labeling, and identification procedure to adequately deal with protozoan sampling and analysis in an international, field setting.

Statistical analysis using the ChiSquare statistic indicates a correlation between the presence of *Cryptosporidium* oocysts and the frequency of diarrheal disease (Likelihood Ratio, ChiSquare = 15.059, $p = 0.0064$), and the presence of *Giardia* cysts and the severity of diarrheal disease (Likelihood Ratio, ChiSquare = 12.157, $p = 0.0327$). ANOVA analysis of the relationship between *Cryptosporidium* oocyst and *Giardia* cyst concentrations and the reported frequency of diarrheal disease indicates a significant correlation between the frequency of diarrheal disease and the concentration of *Cryptosporidium* oocysts (ANOVA, f-ratio = 4.3277, $p = 0.0037$) and *Giardia* cysts (ANOVA, f-ratio = 5.9876, $p = 0.0004$). A correlation between *Cryptosporidium* oocyst and *Giardia* cyst concentrations and average severity of diarrheal disease was also detected, *Giardia* cyst concentrations (ANOVA, f-ratio = 13.4211, $p = 0.0005$) and *Cryptosporidium* oocyst concentrations (ANOVA, f-ratio = 5.3926, $p = 0.0233$).

Introduction

Diarrheal disease continues to be one of the leading causes of morbidity and mortality in the world, WHO (2000). It is responsible for an estimated one-third of all hospitalizations of children less than 5 years of age worldwide, and results in 2.4 -2.9 million deaths within this age group every year, Cama *et al*.(1999). Causes of diarrhea in areas of high prevalence include a wide range of bacteria, viruses, and protozoa such as *Giardia sp.* and *Cryptosporidium sp.,* Gascon *et al*. (2000). Diarrheal diseases, such as cryptosporidiosis and giardiasis, are considered to be the leading cause of morbidity and mortality among children in low-income countries, Levine *et al*.(1986); Snyder and Merson, (1982). Human giardiasis is one of the 10 most frequent infectious diseases in the world, and is categorized as a reemerging infectious agent in many developing countries Ish-Horowicz *et al*. (1989); Thompson *et al*. (2000). *Cryptosporidium sp.* infection is now considered to be one of the leading protozoan causes of diarrhea in the world, Montero *et al*. (2001).

Due to their reliance on drinking water from predominantly shallow wells, children of the Transylvania Region of Romania are at greater risk of consuming water contaminated by chemicals and/or parasites, such as *Giardia* and *Cryptosporidium,* than are children in more developed countries, Ayebo *et al*.(1997). Most Romanian villages in the Transylvania Region are made up of clusters of homes built on small plots, approximately 20 by 40 meters, Zeman (2000). Generally, each home uses a well as a source of water, which is usually located inside the small plot of land on which the home stands.

A previous study conducted in the counties of Alba, Bistrita-Nasaud, Mures, Salaj, and Satu-Mare, all located in the Transylvania Region of Romania, surveyed 70 wells, Zeman (2000). On average, the depths of these wells were approximately 6 meters, with the water table approximately 4 meters below the surface, Zeman (2000). The majority of these wells were lined only down to the water table level, with these linings in various levels of deterioration. Of those surveyed, only 61% of the wells had a casing or protective cover over the wellhead, Ayebo *et al*.(1997); Zeman (2000).

The goal of this current study was to sample and examine the drinking water supplies of 69 of the child participants in the Zeman study for the presence of *Giardia* cysts and *Cryptosporidium* oocysts. A questionnaire was developed to establish the frequency and severity of diarrheal disease episodes in each of the children within the study group to determine if a correlation existed between the presence of *Giardia* cysts and *Cryptosporidium* oocysts and the frequency or severity of diarrheal disease within the study group.

To achieve this goal, procedures had to be established that would quickly and accurately identify the presence of *Giardia* cysts and *Cryptosporidium* oocysts in the water supplies in a field setting where total suspended solids would likely be quite high. The selection of the methods and equipment used for collection, concentration, labeling, and identification of

Giardia cysts and *Cryptosporidium* oocysts was based on their ability to perform efficiently and effectively under projected conditions. Systems chosen were required to meet five major criteria.

Materials and Methods

Questionnaire

A questionnaire was administered to a parent, guardian, or adult family member of each participant, which included questions dealing with the frequency and severity of diarrheal episodes. The questionnaire posed questions concerning the subject's first five years of life. These included questions pertaining to the frequency and severity of diarrheal diseases that occurred during these early years.

Water Sample Collection & Concentration

Sampling of the drinking water used by 69 subjects took place between May 23 and June 12, 2001. Drinking water samples were extracted from the water source using the same method used by the subjects to retrieve water. In most cases, this meant removing the water from a well using a bucket attached to a rope or chain. The water removed from the water source was then poured into a graduated plastic container

The apparatus used to collect the cysts and oocysts from the water sample consisted of an electric, flexible impeller pump, a filter housing and filter module, and laboratory grade rubberized tubing. The pump was equipped to operate using a typical 12-volt automobile battery, allowing for field use of the apparatus. The filter modules were comprised of multiple layers of compressed, open-cell foam discs and a flow rate of 2-3 liters per minute at 30-35 psi (IDEXX Laboratories, catalog number FMC 10601).

When feasible, 20 liters of water from the source was pumped through the filter. In cases were high turbidity was an issue, the volume of water filtered was reduced to 10 liters. Upon completion of the collection process, the collection system was cleaned and flushed for approximately 1-2 minutes using a hypochlorite solution (0.10).

The removal of cysts and oocysts from the filter cartridge was performed using an IDEXX™ wash station (catalog number FMC 10102). An elution procedure as described previously by Sartory *et al*. 1998 was used for all samples. The PBST (phosphate buffered saline solution and tween 20) and recovered particulate material containing any cysts and oocysts were then transferred to 20 mL conical-bottom centrifuge tubes. The solution was then centrifuged for approximately 10 minutes at 5,000x g. The supernatant was then decanted off until a total volume of between 7 and 15 mL was achieved. The pellet was then re-suspended for the staining and identification stages. The elution and concentration of all samples were completed with 72 hours of sample collection.

Cyst and Oocyst Labeling and Identification

An indirect immunofluorescent detection procedure was chosen to aid in the detection of *Giardia* cysts and *Cryptosporidium* oocysts in the water samples. The Hydrofluor™ - Combo system (Strategic Diagnostics Inc. catalog #70810) was used for all labeling procedures.

A cellulose acetate filter (0.22 µm pore size, 25 mm diameter) was soaked in 1x PBS (phosphate buffered saline), pH 7.4, just prior to use. The filter was removed from the PBS only after they lost buoyancy and sank to the bottom of the container. Once removed from the solution, the filter was placed onto a glass frit support (Cole-Parmer catalog number E-02921-00) using membrane filter forceps. The filter was then rinsed with 2 mL of 1% bovine serum albumin (BSA) and allowed to drain.1 mL of concentrated sample was placed on the membrane filter. When turbidity was an issue, this volume was adjusted accordingly to reduce background interference. The membrane filter was then washed with 0.5 mL 1% BSA. The 10x concentrated primary antibody reagent was diluted with normal goat serum (to a final 10%) in 1x PBS. (1/10 volume antibody - 1/10 volume goat serum - 8/10 volume PBS) and 0.5 mL of diluted primary antibody reagent was placed on each membrane filter. The filter was then allowed to incubate for 25 minutes at room temperature. Following the incubation period, the filter was rinsed 5 times with 2 mL 1x PBS. The fluorescence needed to identify *Giardia* cysts and *Cryptosporidium* oocysts was achieved by incubating the sample with a FITC-conjugated antibody (photosensitive labeling reagent) to the murine immunoglobulins (primary antibody). To reduce waste of the labeling reagent, the 10x concentrated labeling reagent was diluted with normal goat serum (to a final 10%) in 1x PBS. Next, 0.5 mL of diluted labeling reagent was added to the membrane filter. The filter was incubated for 25 minutes at room temperature, shielded from light. After the 25 minute incubation period, the filter was rinsed 5 times with 2 mL 1x PBS.

Prior to mounting the filters onto slides for examination, the filter required partial dehydration. This was achieved by sequentially applying 1.9 mL of 10, 20, 40, 80, 90.2% ethanol solutions containing 5% glycerol. Each solution was allowed to dehydrate to a light sheen before applying the next series (without thoroughly drying between applications). Glass slides were labeled and placed in an incubating oven operating at 38º C. 75 µL of 2% dabco-glycerol mounting medium was added to each slide, which were then allowed to warm for 20 - 30 minutes. After 20 - 30 minutes, the slides were removed from the oven, and using the membrane filter with forceps, the membrane filter was placed on a slide. The filter was carefully maneuvered to insure the entire filter was situated on top of the dabco-gyceral mounting medium. After a 20 minute clearing period in the incubation oven, the membrane filter became transparent. An additional 20 µl

mounting medium was added to the center of each membrane filter and covered using a glass coverslip. The slidecover was then sealed to the slide using clear fingernail polish, Strategic Diagnostics (1997). Slides were stored at 2° - 8 ºC in a sealed plastic container, which also contained desiccant. All sample staining was completed within 24 hours of the completion of the concentration procedure.

The membrane filters were examined using an Olympus CH3 epifluorescent microscope within 72 hours. Slides were scanned at 200x magnification, and the cysts and oocysts were identified at 400x magnification. To insure proper identification of the cysts and oocysts was achieved, the following guidelines were followed.
Criteria for identification of *Giardia and Cryptosporidium* cysts included: i.) Must fluoresce an apple-green color; ii.) Must have an oval or round shape; iii.) The size of the suspected cysts must be between 8-18 μm long and 5-15 μm wide for *Giardia* or between 4-7 μm in diameter for *Cryptosporidium;* iv.) The cyst must be whole and rupture free; v.) Visible internal structures consistent with *Giardia* cyst morphological characteristics

Determination of Percent Recovery

The percent recovery of *Giardia* cysts and *Cryptosporidium* oocysts using the selected equipment and procedures was calculated using a commercially prepared solution containing *Giardia* cysts and *Cryptosporidium* oocysts (Biotechnology Frontiers Pty Ltd, catalog number ES-CG100). A total of three runs were performed to determine percent recovery, and the three results were averaged to determine average percent recovery of the sampling process. The average percent recovery of *Giardia* cysts and *Cryptosporidium* oocysts was calculated to be 44% and 38% respectively.

Data Analysis

All statistical analyses were performed using JMP software by SAS Institute, version 4.0.2. Discrete data correlations between the results of the water sampling data and the survey gathered self-reports of severity and frequency of diarrheal disease were tested for significance using the non-parametric Chi square statistics. Continuous data comparisons of *Giardia* cyst and *Cryptosporidium* oocysts concentrations to the survey gathered self-reports of frequency and severity of diarrheal disease were analyzed using the one-way analysis of variance test (ANOVA), followed by tests of significance using Tukey-Kramer HSD. For all comparisons, $p < 0.05$ was considered significant.

Results and Discussion

Of the 69 water sources tested, 27 (39%) were found to be positive for *Cryptosporidium* oocysts, and 30 (43%) positive for *Giardia* cysts. Almost one quarter of the wells (17) tested positive for both *Giardia sp.* and *Cryptosporidium sp*. The average concentration detected of those that tested positive for *Cryptosporidium* oocysts was calculated to be 6.8 oocysts per liter of water. This concentration is twenty-three times higher than the 0.30 oocysts per liter "action level" proposed for *Cryptosporidium* oocysts (Haas and Rose, 1995). The average concentration of *Giardia* cysts was estimated at 4.2 cysts per liter. This level is almost eighty-five times higher than the 0.05 cysts per liter "action level" proposed for *Giardia* cysts (Wallis *et al*., 1996). The highest concentration of *Cryptosporidium* oocysts was estimated at 84.2 oocysts per liter. This sample was taken from a shallow (8 meter), 25 year old well in Gambas, a small village located in Alba county. The well was not covered, and was lined only to a depth of approximately one meter (Zeman, 2000). This same source also produced the highest concentration of *Giardia* cysts detected, calculated to be 36.4 per liter.

Seven (10%) of the 69 subjects reported the frequency of diarrheal disease to be "frequent to very frequent." Of these, 42% used a water source that tested positive for both *Giardia sp.* and *Cryptosporidium sp.*. Thirteen reported the average severity of diarrheal disease to be 3 or higher on a Likert Scale of 1-5 (with 5 being the most severe). Of these subjects, 46% had a water source that tested positive for both protozoa.

Looking strictly at the presence or absence of protozoan in the water, a statistically significant correlation between the presence of *Cryptosporidium* oocysts and the frequency of diarrheal disease was established (Likelihood Ratio, ChiSquare = 15.059, $p = 0.0046$). The correlation between the presence of *Giardia* cysts and the severity of diarrheal disease was also found to be statistically significant (Likelihood Ratio, ChiSquare = 12.157, $p = 0.0327$).

ANOVA analysis of the data indicated a statistically significant correlation between the frequency of diarrheal disease reported and the concentrations of *Giardia* cysts (Figure 1, ANOVA, f-ratio =5.9876, $p = 0.0004$).

Figure 1

Oneway Analysis of *Giardia* Concentrations By Diarrheal Disease Frequency of Attack

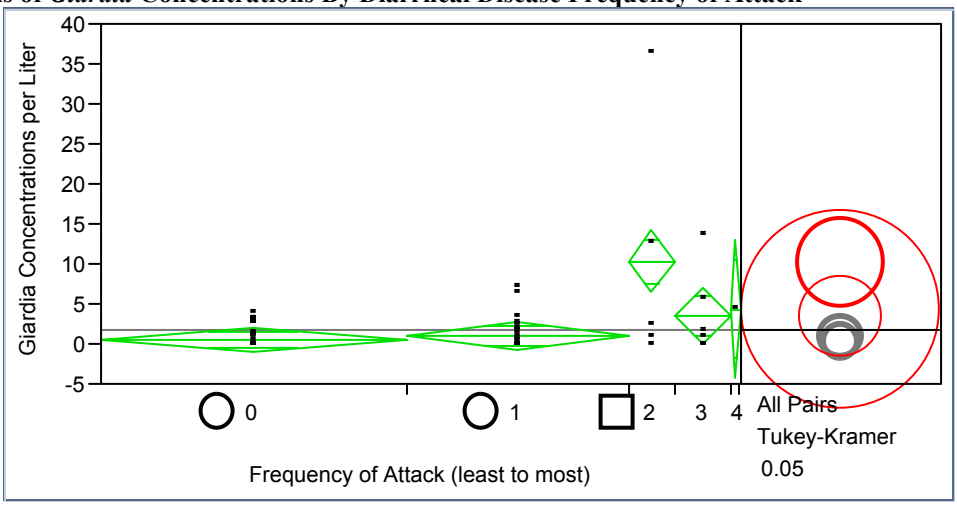

Fig. 1. *Giardia* Concentration vs. Disease Frequency. (ANOVA, f-ratio = 5.9876, $p = 0.0004$)

The diamond represents the 95% confidence interval. The centerline in the diamond indicates the means, and group sample size is indicated by the width of the diamond. An angle of intersection of less than 90° between the comparison circles indicates that the means are significantly different. Groups circled are significantly different from the group that is bordered by a square. If there are no means found significantly different, then none of the groups will be circled or bordered by a square.

The concentrations of *Cryptosporidium* oocysts detected in water samples and the frequency of diarrheal disease was also found to be significant (Figure 2, ANOVA, f-ratio = 4.3277, $p = 0.0037$).

Figure 2

Oneway Analysis of *Cryptosporidium* Concentrations By Diarrheal Disease Frequency of Attack

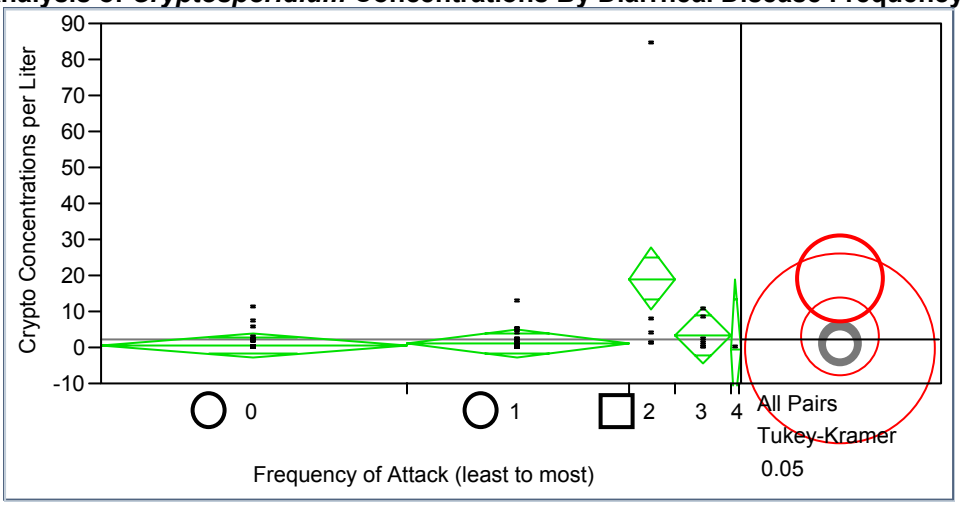

Fig. 2. *Cryptosporidium* Concentration vs. Disease Frequency. (ANOVA, f-ratio = 4.3277, $p = 0.0037$)

Analysis indicates a statistically significant correlation between the severity of diarrheal disease reported and the concentrations of *Giardia* cysts detected in water samples taken from the subject's water source (Figure 3, ANOVA, f-ratio = 13.4211, $p = 0.0005$). Furthermore, there was also established a statistically significant correlation between the concentrations of *Cryptosporidium* oocysts and the severity of diarrheal disease (ANOVA, f-ratio =5.3926, $p = 0.0233$).

A possible explanation for the high prevalence of protozoa in the drinking water, with only moderate levels of diarrheal outbreaks may be at home water treatment. *Giardia* cysts and *Cryptosporidium* oocysts were found to be nonviable if exposed to boiling water for one minute, AWWA (1999). Results of an earlier survey on drinking water practices (Zeman, 2000) found that approximately one third of these households (of the households with

Figure 3
Oneway Analysis of *Giardia* Concentrations By Diarrheal Disease Severity (Mild vs Severe)

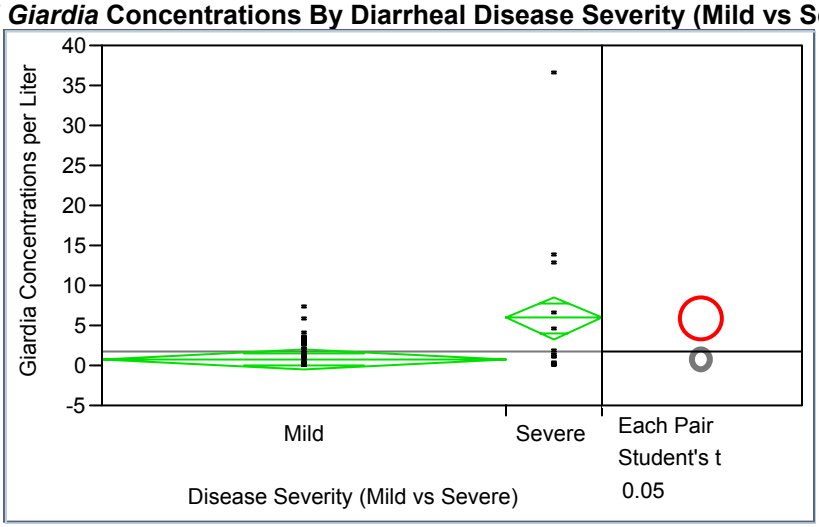

Fig. 3. *Giardia* Concentration vs. Disease Severity. (ANOVA, f-ratio = 13.4211, $p = 0.0005$)

elevated *crypto./giardia* levels) boiled water that was for infant/child consumption, Zeman (2000). When results of water sample analysis for the presence of *Giardia* cysts and *Cryptosporidium* oocysts for those who did not boil the water for infant formula were compared to the reported frequency of diarrheal disease, statistical analysis indicated a statistically significant correlation between the presence of *Cryptosporidium* oocysts and the frequency of diarrheal disease (Likelihood Ratio, ChiSquare = 13.224, $p = 0.0042$). However, there was not a statistically significant correlation found between the comparison of the presence of *Giardia* cysts and *Cryptosporidium* oocysts and the reported frequency of diarrheal disease for those who boiled the water for infant formula (Likelihood Ratio, ChiSquare = 2.246, $p = 0.3254$). Thus at this most vulnerable time of life, the children may have had their exposure to protozoa reduced by water treatment practices within the home. Additionally, while this data is correlational there are other likely sources of contamination including hand/mouth activity in children, the consumption of fresh vegetables/salads, and the presence of farm and domestic animals in the well area and around the home.

Conclusions

- The prevalence of *Cryptosporidium* oocysts (39%) and *Giardia* cysts (43%) in the wells sampled represents a potential health risk for the very young and immunosuppressed individuals living in the rural areas and small villages of the Transylvania region of Romania.
- With one quarter of the wells testing positive for both *Giardia* cysts and *Cryptosporidium* oocysts, it appears that these water supplies are exposed to either animal or human fecal contamination.
- Additional research should be undertaken into the determination of protozoa species and viability found in the study participants' drinking water. This determination of the species of *Giardia* and *Cryptosporidium* present in the water supplies and their viability would allow for a better understanding of exposure levels the study group has experienced. The results of such a study could also shed light onto why the prevalence of severe diarrheal disease is relatively low as compared to the concentrations of *Giardia* cysts and *Cryptosporidium oocysts* detected in the drinking water. It could also give researchers a better understanding of the source and pathways of the fecal contamination in the water supplies.

Acknowledgments

Thanks to the staff at the Romanian Institute of Public Health, located in Cluj-Napoca, Romania for their assistance in obtaining the data presented in this study. A special thanks to Daniela Moga, M.D. and her staff in the Laboratory of Community Environmental Risk Assessment, Romanian Institute of Public Health for supplying laboratory space and equipment as well as their expertise to the American research team. Also, a special thanks to the rest of the American research team; Naomi Bienfang, Bryan Byrd, Randi Huffman, Heather Moore. Their hard work and long hours contributed greatly to the success of this study.

References

AWWA (Ed.). (1999) *Waterborne Pathogens* 1st ed.. American Water Works Association, Denver, CO.

Ayebo, A., Kross, B., Vlad, M. and Sinca, A. (1997) Infant methemoglobinemia in the Transylvania region of Romania. *International Journal of Occupational Health*, **3**, 20-29.

Cama, R., Parashar, D., Taylor, D., Hickey, T., Figueroa, D., Ortega, Y., Romero, S., Perez, J., Sterling, C., Gentsch, J., Gilman, R. and Glass, R. (1999) Enteropathogens and other factors associated with severe disease in children with acute watery diarrhea in Lima, Peru. *The Journal of Infectious Diseases*, **179**, 1139-1144.

Gascon, J., Vargas, M., Schellenberg, D., Urassa, H., Casals, C., Kahigwa, E., Aponte, J., Mshinda, H. and Vila, J. (2000) Diarrhea in children under 5 years of age from Ifakara, Tanzania: a case-control study. *Journal of Clinical Microbiology*, **38**(12), 4459-4462.

Haas, C. and Rose, J. (1995) Developing an action level for *Cryptosporidium*. *Journal of the American Water Works Association*, **87**(9), 81-84.

Ish-Horowicz, M., Korman, S., Shapiro, M., Har-Even, U., Tamir, I., Strauss, N. and Deckelbaum, R. (1989) Asymptomatic giardiasis in children. *The Pediatric Infectious Disease Journal*, **8**(11), 773-779.

Levine, M., Losonsky, G., Herrington, D., Kaper, J., Tacket, C., Rennels, M. and Morris, J. (1986) Pediatric diarrhea: The challenge of prevention. *Pediatric Infectious Disease*, **5**(Suppl.), 29-43.

Montero, J., Sinnott, J., Holt, D. and Lloyd, C. (2001) Biliary cryptosporidiosis: Current Concepts. *Infections in Medicine*, **18**(6), 305-311.

Sartory, D., Parton, A., Roberts, J.and Bergmann, K. (1998) Recovery of *Cryptosporidium* oocysts from small and large volume water samples using a compressed foam filter system. *Letters in Applied Microbiology*, **27**, 318-322.

Strategic Diagnostics Inc. (1997) *Hydrofluor-Combo: Indirect immunofluorescent detection procedure for Giardia cysts and Cryptosporidium oocysts*. Retrieved September 18, 2001 from the World Wide Web: http://www.sdix.com/FTPfiles/wqfoodag/hydrflurpkg.pdf

Snyder, S. and Merson, M. (1982) The magnitude of the global problem of acute diarrheal disease. A review of active surveillance data. *Bulletin of the World Health Organization,* **66**, 605-613.

Thompson, R., Hopkins, R. and Homan, L. (2000) Nomenclature and genetic groupings of *Giardia* infecting mammals. *Parasitology Today*, **16**(5), 210-213.

Wallis, P., Erlandsen, S., Isaac-Renton, J., Olsen, M., Robertson, W. and VanKeulen, H. (1996) Prevalence of *Giardia* cysts and *Cryptosporidium* oocysts and characterization of *Giardia spp*. isolated from drinking water in Canada. *Applied and Environmental Microbiology*, **62**(8), 2789-2797.

WHO (Ed.) (2000) *The World health report 2000.* World Health Organization, Geneva.

Zeman, C. (2000) Exposure assessment methodology developed in support of a pilot study of long-term neuropsychological impacts of methemoglobinemia and high nitrate exposure in infants of Transylvania, Romania with an added case/control study of methemoglobinemia risk factors. UMI: Ann Harbor, Michigan.