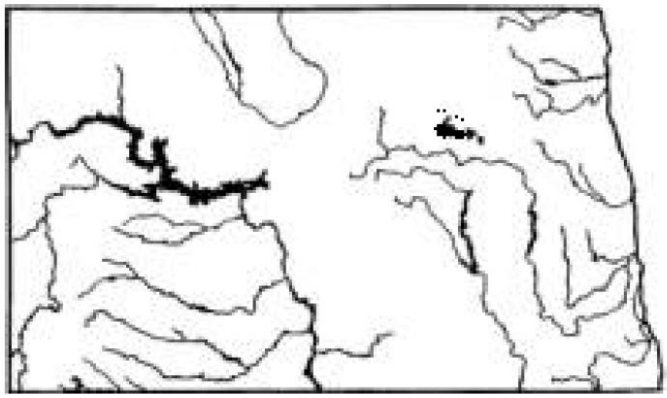


Technical Report No: ND12-07

Source Tracking of Cryptosporidium in the Red River Valley

Brianna L.S. Stenger[1], Mark E. Clark[2], John M. McEvoy[3]
NDWRRI Fellow[1]
Associate Professor, Dept. of Biological Sciences[2]
Associate Professor, Dept. of Veterinary and Microbiological Sciences[3]
North Dakota State University
Fargo, North Dakota

June 2012

North Dakota Water Resources Research Institute
North Dakota State University, Fargo, North Dakota

Technical Report No: ND12-07

Source Tracking of Cryptosporidium in the Red River Valley

Brianna L.S. Stenger[1], Mark E. Clark[2], John M. McEvoy[3]
NDWRRI Fellow[1]
Associate Professor, Dept. of Biological Sciences[2]
Associate Professor, Dept. of Veterinary and Microbiological Sciences[3]
North Dakota State University
Fargo, North Dakota

June 2012

The work upon which this report is based was supported in part by federal funds provided by the United States of Department of Interior in the form of ND WRRI Graduate Research Fellowship for the graduate student through the North Dakota Water Resources Research Institute.

Project Period: March 1, 2009 - February 28, 2012
Project Number: 2009ND183B

North Dakota Water Resources Research Institute
Director: G. Padmanabhan
North Dakota State University
Fargo, North Dakota 58108

Table of Contents

List of Figures & Tables

Abstract

Cryptosporidium is a genus of ubiquitous parasites found in humans, livestock, wildlife, and water sources. Many cryptosporidia are host adapted or have a limited host range, and this information can be exploited to identify sources and public health significance of water contamination. We collected water samples from the Red River and its large tributaries including the Buffalo, Maple, Rush, Sheyenne and Wild Rice Rivers. The samples were processed in accordance with EPA Method 1622/1623. We also collected samples and characterized *Cryptosporidium* from various mammals in the Red River Valley. Our data show that both livestock and wildlife contribute to *Cryptosporidium* contamination in water, and cattle adapted *C. andersoni* is the most prevalent species. Most of the *Cryptosporidium* species identified in rivers are not considered to be major human pathogens. However, the high prevalence of cattle adapted species would indicate that other cattle associated zoonotic pathogens could also be present.

Acknowledgements

We would especially like to thank Christopher Pennil, Cathy Giddings, and Vince Lanctot for assisting with water collection, filtration, DNA extraction, and slide preparation. We acknowledge the funding and fellowship support provided by United States Department of Agriculture, National Institute of Health, and the North Dakota Water Resources Research Institute.

Objective

To identify the source and public health importance of *Cryptosporidium* contamination in the Red River and its tributaries.

Background

The genus *Cryptosporidium* is composed of over 60 different types of ubiquitous parasites. *Cryptosporidium* parasites are known to infect more than 150 species including fish, amphibians, reptile, birds, and mammals, and were not recognized as a potential public health threat until the later part of the 20th century (Fayer 2004). These parasites are commonly found in surface waters, but our understanding of how the water supply gets contaminated and the public health significance is quite poor. Recent advances in molecular typing have allowed us to narrow down the source of contamination and assess the public health significance of contaminated water. Many cryptosporidia are considered host-adapted or have a limited host range (Xiao et al. 2004; Xiao et al. 2002) and this can be exploited to identify the source of water contamination.

Transmission occurs through contact with infected animal or human fecal material. Routes of transmission can be from human to human, animal to animal, human to animal, or animal to human, waterborne, or foodborne. Waterborne transmission is of greatest public health concern because oocysts (the environmental stage) are resistant to conventional disinfection practices such as chlorination (Dawson 2003). Waterborne outbreaks are can cause widespread cryptosporidiosis, which can be deadly in immunocompromised individuals. One of the most notable outbreaks occurred in Milwaukee, Wisconsin, during the spring of 1993. More than 400,000 people were infected with *Cryptosporidium* and over 100 people died. Unfortunately at the time, the source of the *Cryptosporidium* could not be definitively traced but possibilities include run-off, cattle along the rivers, slaughterhouses, human sewage, or a combination of events (Mac Kenzie et al. 1994). The estimated cost of the outbreak was $96.2 million, $64.6 of that in productivity losses and $31.7 million in medical costs (Corso et al. 2003). State and local governments along with the Environmental Protection Agency (EPA) have taken measures to reduce the chance of such an outbreak again by following the Long Term 2 Enhanced Surface Water Treatment Rule (CDC).

While *Cryptosporidium* oocysts are common in water supplies, only a few of the species/genotypes are known to pose any significant threat to human health. The two main species infecting humans are *Cryptosporidium parvum* and *Cryptosporidium hominis* (Zhou et al. 2003; McLauchlin et al. 2000). *Cryptosporidium hominis* almost exclusively infects humans, whereas *C. parvum* can be found in a broad range of hosts including humans, rodents, and calves. *Cryptosporidium parvum* has been responsible for a number of outbreaks of cryptosporidiosis in humans worldwide, and has been a common cause of sporadic outbreaks in Minnesota and Wisconsin (Feltus et al. 2006; Pennil et al. 2007).

Most *Cryptosporidium* research has focused on *Cryptosporidium* in humans and livestock. Relatively few studies have looked at the role wildlife can play in the transmission of *Cryptosporidium* although many believe that wildlife are important contributors of environmental contamination along with humans and livestock (Appelbee et al. 2005). A New York watershed study found that fur-bearing mammals excrete host-adapted cryptosporidia that are not known to be of significant public health threat (Jiang et al. 2005). A study in a New York City watershed looked at factor such as age, season, habitat, and land use on the prevalence of *Cryptosporidium* in wildlife. They did find wildlife-adapted *Cryptosporidium* and the pathogenic *C. parvum*, so wildlife could play a role in zoonotic transmission (Ziegler et al. 2007).

Our objective in this study was to identify the *Cryptosporidium* species and genotypes in the Red River and its tributaries and in various mammals inhabiting the watershed. The data collected, together with our understanding of the host adapted nature of many types of *Cryptosporidium*, will be used to determine the public health significance of *Cryptosporidium* in the region.

Methods

Water Sampling

A total of 30 flood water samples were collected along the Red River or the North, Rush River, Maple River, Wild Rice River, Buffalo River, and the Sheyenne diversion in West Fargo during the spring flooding in 2009 and 2010. Our research group previously collected 28 water samples in 2007 and 2008 along the Buffalo River near Hawley, MN (Pennil et al. 2009). Approximately 20L of water were filtered for each water sample through Envirocheck HV filter

capsules (Pall Gelman Laboratory, MI) and analyzed in accordance with EPA Method 1622/1623 *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA (EPA 2001). *Cryptosporidium* was isolated from pelleted samples by immunomagentic separation (IMS) using a Dynal kit. The IMS purified samples were then used for molecular analyses or enumerated (only samples from 2010).

Mammal Fecal Samples

Sherman box traps (H.B. Sherman Traps Inc., Tallahassee, FL) and Tomahawk (Tomahawk Live Trap Company, Tomahawk, WI) live traps were set near waterways to live-capture targeted small wild mammals including mouse, vole, shrew, and squirrel species. All traps were baited, set, and checked daily for a 4-5 day trapping session. Multiple trapping sessions were conducted per year at each site. Captured animals were carefully taken from the traps, and fecal samples were collected from inside the trap or fresh samples directly from the animal. Animals were weighed, sexed, ear tagged and released. Ear tags allowed for identification of individual animals and would eventually be used for mark-recapture density estimates. Animals that expired in the traps were brought back to the lab and dissected to clear out the contents of the intestines.

DNA Extraction

DNA was extracted from the IMS bead-*Cryptosporidium* oocyst mixtures using a QIAamp DNA mini kit (QIAGEN, Valencia, CA). Initially, 180 ul of Buffer ATL was added to the bead-oocyst mixture and then subjected to five freeze-thaw cycles (liquid nitrogen 56*C and 65* water bath) for 2 and 5 min, respectively. Subsequent steps were performed following the manufacturer's instructions.

DNA was extracted from 100-200 µg fecal samples by alkaline digestion and phenol-chloroform extraction, and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) as previously described (Peng et al. 2003) Briefly, 66.6 µl of 1 M KOH and 18.6 µl of 1 M DTT (Dithiothreitol) were added to a 1.5 µl centrifuge tube containing 100-200 µl of stool. The samples were incubated at 65°C for 15 minutes, neutralized with 8.6 µl of 25% HCL and buffered with 160 µl of 2 M Tris-HCL (pH 8.3). DNA was extracted with 250 µL of phenol:chloroform:isoamyl alcohol (Invitrogen, Carlsbad, CA), mixed and centrifuged at 3330 × g for 5 min. (Thermo Scientific Heraeus Pico 21 centrifuge). The supernatant was removed to a 2.0 ml Eppendorf tube containing 1.0 ml of buffer ASL from the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). The DNA was further purified in accordance with manufacturer's instructions and stored at -20°C until required for PCR.

Enumeration of Oocysts

IMS purified and dissociated oocysts from water samples were labeled with a FITC conjugated anti-*Cryptosporidium* monoclonal antibody, Crypt-a-Glo™ (Waterborne Inc., LA), and mounted on Dynal glass slides. An Olympus BX61 microscope under 400x magnifcation with epifluorescence illumination was used to count oocysts. Some of the enumerated samples were also used for molecular analysis and prepared as described by (Ruecker et al. 2005).

Identification of *Cryptosporidium*

Identification of *Cryptosporidium* species/genotypes was determined by amplifying an approximately 830bp fragment of the small subunit (SSU) rRNA gene using a nested PCR procedure as previously described by (Xiao et al. 1999;Xiao et al. 2000;Feltus et al. 2006). The final products were purified using the Wizard SV gel clean-up system (Promega, Madison, WI) and bidirectionally sequenced on an Applied Biosystems 3730xl DNA analyzer (Applied

Biosystems, Foster City, CA) using the secondary primers. The sequences were compared to non-redundant sequences in GenBank using the BLAST algorithm to determine the *Cryptosporidium* species/genotype.

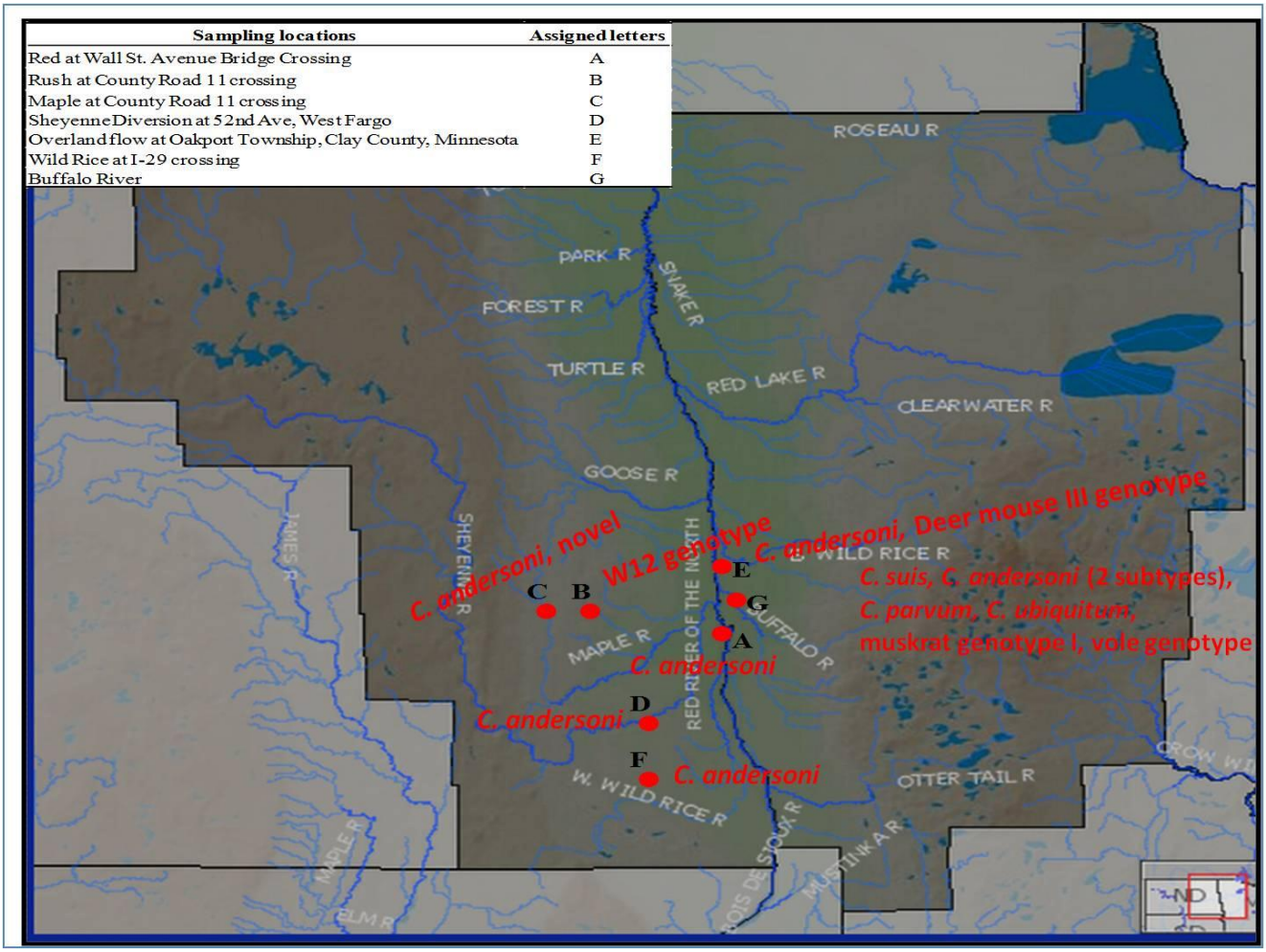
Results

Cryptosporidium in Water Samples

Cryptosporidium was detected in 23/30 (77%) water samples collected during spring flooding in 2009 and 2010. In 2009, 9/13 (69%) water samples were positive. We identified *C. andersoni* in 7 samples, C. suis in 1 sample, and Deer mouse genotype III in 1 sample. We detected *Cryptosporidium* in 14/17 (82%) water samples in 2010 and identified W12 genotype in one sample and *C. andersoni* in 3 samples. Our research group previously detected muskrat genotype I, vole genotype, *C. parvum*, *C. ubiquitum*, and 2 subtypes of *C. andersoni* in the Buffalo River. Figure 1 is a map of the sampling site locations and types of cryptosporidia from each location.

The average number of oocysts was determined for samples from the 2010 Flood. We estimated 1.3 oocysts per liter during the crest and .3 oocysts per liter one week later. Based on an approximate flow of 560,000L per second (USGS), the flow of oocysts was calculated at 728,000 per second.

Figure 1. Locations of sampling sites along with the *Cryptosporidium* associated at each location/river. Adapted from Wadhawan et al.



<u>*Cryptosporidium* in Mammals</u>

A total of 109/282 (38.7%) small mammal samples (wildlife species) tested positive for *Cryptosporidium*. The host species included Northern short-tailed shrews (*Blarina brevicauda*), Southern red-backed voles (*Clethrionomys gapperi*), *Peromyscus* spp. (deer mice and white-footed mice), Eastern grey squirrels (*Sciurus carolinensis*), Eastern fox squirrels (*Sciurus niger*), American red squirrels (*Tamiasciurus hudonicus*), and meadow voles (*Microtus pennsylvanicus*). Meadow jumping mice (*Zapus hudsonius*) and thirteen-lined ground squirrels (*Spermophilus*

tridecemlineatus) were also trapped, but did not test positive for *Cryptosporidium*. A total of 11 different types of cryptosporidia were detected in the small mammals. *C. suis* was the only species found in water and not in mammal samples; whereas Muskrat II, Fox, Shrew, Skunk, and Deer mouse genotypes were found in mammal samples but not water samples.

Tables 1, 2, and 3 indicate the *Cryptosporidium* and corresponding host species trapped along the Buffalo River (previous work adapted from Pennil et al. 2009), near the Red River, and along the Red River, respectively. Table 4 is a summary of the *Cryptosporidium* associated with each host and highlights the types of *Cryptosporidium* found in both mammal and water samples.

Table 1. A 13/26 (50%) occurrence of *Cryptosporidium* along the Buffalo River (adapted from Pennil et al. 2009)

Host Species	***Cryptosporidium* species/genotype**
Meadow vole	Fox genotype, Muskrat genotype I, Muskrat genotype II, Vole genotype
Northern short-tailed shrew	Muskrat genotype I
Southern red-backed vole	Fox genotype, Muskrat genotype I
Peromyscus spp.	Deer mouse genotype, Deer mouse genotype III
Red squirrel	*positive but could not sequence

Table 2. A 60/148 (40.5%) occurrence of *Cryptosporidium* near the Red River

Host Species	***Cryptosporidium* species/genotype**
Grey squirrel	*C. parvum*, *C. ubiquitum*, Deer mouse genotype III, Skunk genotype
Southern red-backed vole	Muskrat genotype II
Peromyscus spp.	*C. ubiquitum*, Deer mouse genotype III, Muskrat genotype II
Red squirrel	*C. ubiquitum*, Deer mouse genotype III

Table 3. A 36/108 (33.3%) occurrence of *Cryptosporidium* along the Red River

Host Species	***Cryptosporidium* species/genotype**
Meadow vole	*C. parvum*, Muskrat genotype II, W12 genotype,
Northern short-tailed shrew	Shrew genotype
Fox squirrel	*C. ubiquitum*
Peromyscus spp.	Deer mouse genotype II, Muskrat genotype II
Red squirrel	*C. parvum*, *C. ubiquitum*, Skunk genotype
Grey squirrel	*C. parvum*, *C. ubiquitum*, Skunk genotype

Table 4. A summary of the *Cryptosporidium* associated with each host. Those in red indicate types of *Cryptosporidium* also found in water samples.

Host Species	***Cryptosporidium* species/genotype**
Meadow vole	*C. parvum*, Fox genotype, Muskrat I genotype, Muskrat II genotype, Vole genotype, W12 genotype,
Northern short-tailed shrew	Muskrat I genotype, Shrew genotype
Fox squirrel	*C. ubiquitum*
Peromyscus spp.	*C. parvum*, *C. ubiquitum*, Deer mouse genotype, Deer mouse III genotype, Muskrat II genotype
Red squirrel	*C. parvum*, *C. ubiquitum*, Skunk genotype
Grey squirrel	*C. parvum*, *C. ubiquitum*, Deer mouse III genotype, Skunk genotype
Southern red-backed vole	Fox genotype, Muskrat I genotype, Muskrat II genotype

Conclusions

Cryptosporidium parasites are ubiquitous, and we identified a number of different types cryptosporidia in wildlife hosts, and water sources during periods of flooding and non-flooding in the Red River Valley. We determined that flooding and run-off can contribute to the movement and spread of *Cryptosporidium* from land to river systems. Livestock and wildlife host species both contributed to the *Cryptosporidium* contamination of water systems the Red River Valley. *C. andersoni* was the most prevalent type of *Cryptosporidium* found in water samples, but is host-adapted and rarely found to infect humans (Xiao et al. 2002). *C. suis* was also found in a water sample, but is another host-adapted species of *Cryptosporidium* that infects swine (Ryan et al. 2004). Two species, *C. parvum* and *C. ubiquitum* are known human pathogens that warrant some caution. Both species were found in water and wildlife samples. *C. parvum* is also known to infect calves and can be a causative agent of diarrheal disease or scours (Santin et al. 2004). We do not believe these species are a major concern in the Red River Valley for a number of reasons. One, the water samples and wildlife positive for *C. parvum* and *C. ubiquitum* were not found in the same area. Two, the two species of *Cryptosporidium* were not commonly seen in either water or wildlife samples. Three, water treatment plants have implemented the EPA's Long-Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) which was enacted to reduce the incidence of pathogenic microorganisms (like *Cryptosporidium*) that may cause waterborne diseases. Water treatment plants are well aware of the potential problems *Cryptosporidium* can cause, and are well equipped to minimize the risk of *Cryptosporidium* in our drinking water. Generally, the *Cryptosporidium* isolated from wildlife samples and found in the water pose little threat to human health.

References from the project:

- Stenger, B.L.S., Pennil, C.C., Lanctot, V.T., Giddings, C.W., Clark, M.E., and McEvoy, J.M. "Source tracking of *Cryptosporidium* spp. in the Red River Valley" North Dakota Water Quality Monitoring Conference, Bismarck, ND, 2012.
- Stenger, B.L.S., Lanctot, V. Clark, M.E., Giddings, C.W., McEvoy, J.M. "*Cryptosporidium* in small mammals from North Dakota, Minnesota, and Tennessee" Joint annual meetings of the North Dakota and Minnesota Wildlife Societies, Fargo, ND, 2012.
- Stenger, B.L., Pennil, C. C., Clark, M.E., Lanctot, V. , Giddings, C.W., and McEvoy, J.M. 2012. "Source tracking of *Cryptosporidium* spp. in the Red River Valley" Joint annual meetings of the North Dakota and Minnesota Wildlife Societies, Fargo, ND, 2012.
- Stenger, B.L.S., Pennil, C.C., Lanctot, V., Giddings, C., Clark, M., McEvoy, J.M. *Cryptosporidium* genotypes infecting small mammals. 2011. North Dakota and South Dakota joing EPSCoR conference, North Dakota State University, Fargo, ND.
- Stenger, B.L., Pennil, C. C., Clark, M.E., Lanctot, V. , Giddings, C.W., and McEvoy, J.M. 2011 "*Cryptosporidium* genotypes in small mammal populations" 8th Northern Plains Biological Sciences Symposium in Grand Forks, ND, 2011
- McEvoy J.M. and Clark, M.E. 2010. Studying the source, fate, and transport of *Cryptosporidium*. NDSU College of Science and Math, Annual Newsletter, p 6.
- Stenger, B. L. S., Pennil, C., Clark, M., Lanctot, V., Giddings, C., and McEvoy, J.M. *Cryptosporidium* Transport in the Red River Valley during Major Spring Floods. North Dakota EPSCoR Conference, Grand Forks, September 30, 2010.

- Pennil, C.C., Clark, M.E., Schneck, B.L., Lanctot, V., Giddings, C.W. and McEvoy, J.M. A great northern migration: *Cryptosporidium* in the Red River Valley during major spring floods. National Synthesis Workshop: Pathogens (Bacteria, Viruses and Protozoa) in Rural and Agricultural Water and Watersheds. Ithaca, New York. May 16-20, 2010.
- Schneck, B.L., Pennil, C. C., Clark, M.E., Lanctot, V., Giddings, C.W., and McEvoy, J.M. 2010 "*Cryptosporidium* transport in the Red River Valley during major spring floods" Summer COBRE poster presentation in Fargo, ND, 2010.
- Schneck, B.L., Pennil, C.C., Lanctot, V., Giddings, C.W., Clark, M.E., McEvoy, J.M. *Cryptosporidium* genotypes in Midwestern mammals. National Water Quality Conference, Hilton Head, South Carolina, February 20-25, 2010
- Pennil, C.C., Clark, M.E., Schneck, B.L., Giddings, C.W. and McEvoy, J.M. High prevalence of *Cryptosporidium andersoni* in surface water during a major spring flooding event. National Water Quality Conference, Hilton Head, South Carolina, February 20-25, 2010.

Citations

Appelbee,A.J., Thompson,R.C. and Olson,M.E. (2005) *Giardia* and *Cryptosporidium* in mammalian wildlife--current status and future needs. *Trends in Parasitology* **21**, 370-376.

Corso,P.S., Kramer,M.H., Blair,K.A., Addiss,D.G., Davis,J.P. and Haddix,A.C. (2003) Cost of illness in the 1993 waterborne *Cryptosporidium* outbreak, Milwaukee, Wisconsin. *Emerging Infectious Diseases* **9**, 426-431.

Dawson, D. (2003) *Foodborne Protozoan Parasites*.

EPA (2001) *Method 1623: Cryptosporidium and Giardia in Water Filtration/IMS/Fa*.

Fayer,R. (2004) *Cryptosporidium*: a water-borne zoonotic parasite. *Veterinary Parasitology* **126**, 37-56.

Feltus,D.C., Giddings,C.W., Schneck,B.L., Monson,T., Warshauer,D. and McEvoy,J.M. (2006) Evidence supporting zoonotic transmission of *Cryptosporidium* spp. in Wisconsin. *J. Clin. Microbiol.* **44**, 4303-4308.

Jiang,J., Alderisio,K.A. and Xiao,L. (2005) Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. *Applied and Environmental Microbiology* **71**, 4446-4454.

Mac Kenzie,W.R., Hoxie,N.J., Proctor,M.E., Gradus,M.S., Blair,K.A., Peterson,D.E., Kazmierczak,J.J., Addiss,D.G., Fox,K.R., Rose,J.B. and . (1994) A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. *New England Journal of Medicine* **331**, 161-167.

McLauchlin,J., Amar,C., Pedraza-Diaz,S. and Nichols,G.L. (2000) Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal samples from livestock animals. *Journal of Clinical Microbiology* **38**, 3984-3990.

Peng,M.M., Wilson,M.L., Holland,R.E., Meshnick,S.R., Lal,A.A. and Xiao,L. (2003) Genetic diversity of *Cryptosporidium* spp. in cattle in Michigan: implications for understanding the transmission dynamics. *Parasitology Research* **90**, 175-180.

Pennil, C. C., Giddings, C. W. and McEvoy, J. M. (2007) *Cryptosporidium parvum* subgenotypes from humans and calves in Minnesota. The 2nd International Conference of *Giardia* and *Cryptosporidium*, Morelia, Mexico.

Pennil, C. C., Clark, M.E., Giddings, C. W., Schneck, B.L, and McEvoy, J. M. (2009) Determing sources of *Cryptosporidium* in a rural watershed. Master's Thesis, North Dakota State University, Fargo, ND.

Ruecker,N.J., Bounsombath,N., Wallis,P., Ong,C.S., Isaac-Renton,J.L. and Neumann,N.F. (2005) Molecular forensic profiling of *Cryptosporidium* species and genotypes in raw water. *Appl. Environ. Microbiol.* **71**, 8991-8994.

Ryan,U.M., Monis,P., Enemark,H.L., Sulaiman,I., Samarasinghe,B., Read,C., Buddle,R., Robertson,I., Zhou,L., Thompson,R.C. and Xiao,L. (2004) *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (*Sus scrofa*). *The Journal of Parasitology* **90**, 769-773.

Santin,M., Trout,J.M., Xiao,L., Zhou,L., Greiner,E. and Fayer,R. (2004) Prevalence and age-related variation of *Cryptosporidium* species and genotypes in dairy calves. *Veterinary Parasitology* **122**, 103-117.

Wadhawan, T., Stenger, B., Pennil, C., Clark, M., Giddings, C., and McEvoy, J. Molecular characterization and enumeration of Cryptopsoridium prevelenct in the Red River of the North during two consecutive spring flooding events. Manuscript in preparation.

Xiao,L., Alderisio,K., Limor,J., Royer,M. and Lal,A.A. (2000) Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. *Applied and Environmental Microbiology* **66**, 5492-5498.

Xiao,L., Fayer,R., Ryan,U. and Upton,S.J. (2004) *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clinical Microbiology Reviews* **17**, 72-97.

Xiao,L., Morgan,U.M., Limor,J., Escalante,A., Arrowood,M., Shulaw,W., Thompson,R.C., Fayer,R. and Lal,A.A. (1999) Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. *Applied and Environmental Microbiology* **65**, 3386-3391.

Xiao,L., Sulaiman,I.M., Ryan,U.M., Zhou,L., Atwill,E.R., Tischler,M.L., Zhang,X., Fayer,R. and Lal,A.A. (2002) Host adaptation and host-parasite co-evolution in *Cryptosporidium*: implications for taxonomy and public health. *International Journal for Parasitology* **32**, 1773-1785.

Zhou,L., Singh,A., Jiang,J. and Xiao,L. (2003) Molecular surveillance of *Cryptosporidium* spp. in raw wastewater in Milwaukee: implications for understanding outbreak occurrence and transmission dynamics. *Journal of Clinical Microbiology* **41**, 5254-5257.

Ziegler,P.E., Wade,S.E., Schaaf,S.L., Stern,D.A., Nadareski,C.A. and Mohammed,H.O. (2007) Prevalence of *Cryptosporidium* species in wildlife populations within a watershed landscape in southeastern New York State. *Veterinary Parasitology* **147**, 176-184.