Understanding Risk Factors for Cryptospordiosis

Project Scope

Oocysts of the enteric protozoa *Cryptosporidium parvum* are ubiquitous in the aquatic environment. Previous taxonomic classification of *C. parvum* included two genotypes of public health concern: Genotype 1 that only infects humans, and Genotype 2 that occurs in a wide variety of animals, including humans. Although small numbers of *C. parvum* oocysts are frequently detected in treated drinking water, it is not known whether they constitute a sufficient dose to cause disease (cryptosporidiosis) in exposed humans. There is a need to assess and determine the actual public health risk associated with consumption of drinking water contaminated with small numbers of *C. parvum* oocysts—especially for sensitive populations such as immunosuppressed persons. The limitations imposed on using human subjects for this research however, necessitates the use of animal models that are similar to humans in terms of risk susceptibility and host response to *Cryptosporidium*.

Existing data on *C. parvum* susceptibility comes primarily from rodents, which differ physiologically from humans in important ways, and models that more closely replicate human responses are needed. To this end, this project utilized a gnotobiotic pig model (i.e., pigs rendered free of microbiological contaminants and into which known microorganisms can be introduced for research purposes) to assess risk as a function of specific host factors suspected of increasing (or decreasing) susceptibility to disease following *C. parvum* oocyst exposure. The overall goal was to increase knowledge and understanding of the risks associated with cryptosporidiosis. The three specific research project objectives were to:

Grant Title and Principal Investigator

Understanding Risk Factors to *Cryptosporidium parvum*: Studies in Gnotobiotic Pigs (EPA Grant #R826138)

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Key Findings and Implications

Analytical Accomplishments:

- The virulence of *Cryptosporidium parvum* in the gnotobiotic pig model is dependent on the *C. parvum* strain (parental versus clone; Genotype 1 versus Genotype 2), the dose or number of oocysts ingested, as well as the age and immune status of the pig.
- The availability and use of single-oocyst "cloned" *C. parvum* isolates appears to eliminate both genetic and biological behavior variability (e.g., differences in infectivity) that often is observed within and between different pools of the same "uncloned" *C. parvum* strain. This represents a significant advancement for *Cryptosporidium* research.
- Dual infection studies failed to produce genetic recombinants of Genotype 1 and Genotype 2 *C. parvum*. Once an infection with one genotype was established, superimposing infections by the other genotype were not observed, regardless of the strain or dose given.

Implications of Research and Impacts of Results:

- Demonstrated that the neonatal gnotobiotic pig is an appropriate model for comparative studies of infection with Genotype 1 and Genotype 2 *C. parvum*.
- Taken collectively, this research provides support for designation of the new species, *C. hominis* for Genotype 1 *C. parvum*.

Publications include 8 peer reviewed journal articles, 2 book chapters, and 13 published abstracts from meetings, conferences, and workshops.

Project Period: February 1998 to February 2001 (extended to December 2002)

1. Determine the infectivity and virulence of different *Cryptosporidium* strains known to infect humans in gnotobiotic pigs.
2. Assess the impact of host age and immune status on cryptosporidiosis in the model system; and
3. Characterize the porcine host immunological responses to *Cryptosporidium* infection.

Relevance to ORD's *Drinking Water Research Multi-Year Plan* (2003 Edition)

This project contributes to two of three Long-term goals for drinking water research: (1) By 2010, develop scientifically sound data and approaches to assess and manage risks to human health posed by exposure to regulated waterborne pathogens and chemicals, including those addressed by the Arsenic, M/MDP, and Six-Year Review Rules; and (3) By 2009, provide data, tools and technologies to support management decisions by the Office of Water, state, local authorities and utilities to protect source water and the quality of water in the distribution system.

This research increases knowledge and understanding of risks associated with *Cryptosporidium*—a waterborne pathogen of key public health and regulatory importance—through assessment of specific host factors that affect the likelihood of developing cryptosporidiosis. The grant has successfully established the gnotiobiotic pig model as an alternative tool for diagnostic screening of water samples for determination of permissible limits of this enteric pathogen in public water supplies. This animal model can also be used for amplification of *C. parvum* isolates from specimens that are of limited quantity or contain too few oocysts to ensure that adequate amounts of these protozoa are available for study. The findings from this research help provide the foundation for evaluating the impact of immune factors in risk assessment which reduces uncertainty associated with inter-species differences in susceptibility thereby making risk assessments more accurate. Lastly, the findings provide the biological evidence that Genotype 2 and 1 *C. parvum* are, indeed, separate and distinct species (*C. parvum* and *C. hominis,* respectively).

To carry out this research, an extensive series of *in vivo* studies were conducted in conjunction with *in vitro* laboratory experiments. The results and implications of some of the major studies are summarized below in their approximate order of completion.

Project Results and Implications

Genotyping/Subgenotyping Studies: The grant's initial studies used the Grafton Compton Human I (GCH1) *C. parvum* isolate originating from an AIDS patient and the Ohio (OH) *C. parvum* isolate originating from an immunocompetent adult laboratory worker with clinical cryptosporidiosis. Molecular characterization showed both strains to be Genotype 2 *C. parvum*. The research team was able to isolate several additional Genotype 2 *C. parvum* strains, other *Cryptosporidium* species strains that can infect humans, and several strains of human Genotype 1 (HuG1) *C. parvum*. Most of these isolates were successfully propagated and maintained over the course of this study in gnotobiotic pigs for use in infectivity experiments that are summarized below. Furthermore, "clones" of GCH1 and OH (and several other *C. parvum*) strains were successfully derived using micromanipulation techniques to isolate a single oocyst of each strain, which was subsequently inoculated into gnotobiotic pigs for viable population maintenance and use throughout the project.

Infectivity of Genotype 1 *C. parvum*, Genotype 2 *C. parvum*, and Non-Human *Cryptosporidium* Species: This series of experiments assessed the infectivity of parental and cloned *Cryptosporidium* oocysts given to neonatal (1 to 4 day old) gnotobiotic pigs. More specifically, the research team attempted to determine/estimate the minimum infective dose (MID), as measured by the presence of (mild) diarrhea and an extended period of oocyst shedding; the median (50 percent) diarrhea dose (DD_{50}) which results in mild to moderate diarrhea for 5 to 10 days; and the median lethal dose (LD_{50}) for each isolate. Comparative infection studies using parental oocysts of each isolate suggested differences between their DD_{50}s and LD_{50}s but not their MIDs (all $\leq$ 5 oocysts). Single oocyst clones of GCH1 and OH (Genotype 2) *C. parvum* were subsequently tested for their infectivity in neonatal gnotobiotic pigs. Like their parental stock, the MID for each clone was low, in fact, a single oocyst. However, the DD_{50} and LD_{50} for freshly propagated (< 3 weeks of age) oocysts of both GCH1 and OH clones were shown to be far lower at $\leq$ 5 oocysts. Furthermore, variability in the clinical responses of gnotobiotic pigs to different pools of

freshly propagated *C. parvum* clones was minimal to nonexistent, suggesting that the clones were a genetically homogeneous (clonal) population of parasites, as compared to the parental stock. Based on these experiments, the researchers concluded that isolates of Genotype 1 *C. parvum*, Genotype 2 *C. parvum*, and one (of three tested) non-human species (*C. meleagridis*) readily infect neonatal gnotobiotic pigs and demonstrates the utility of this animal model for comparative studies of *C. parvum* infectivity.

Dual Infection Studies with Genotype 2 and Genotype 1 *C. parvum*: One day-old pigs were inoculated with both Genotype 2 (GCH1) oocysts and Genotype 1 (HuG1) oocysts and sacrificed to evaluate tissues and fecal contents. In all but one instance, repeated molecular analysis of the small and large intestinal contents revealed only Genotype 2 DNA while genetic recombinations of GCH1 and the HuG1 strain were not found. Because *C. parvum* Genotype 2 oocysts appeared to out-compete Genotype 1, a second experiment was performed in which HuG1 infection was established followed by inoculation with GCH1 *C. parvum*. This time, repeated molecular analysis of the intestinal contents revealed only the human genotype, and no genetic recombinations were found. Findings from these studies provide scientific evidence to support designation of the new species *C. hominis* for the Genotype 1 *C. parvum* (see more below).

Immune Status and Cryptosporidiosis: Experiments to this point in the research project clearly demonstrated that the status of the pig's immune system (i.e., immunocompetent versus immunosuppressed) could not be affecting the risk of infection, since the MID in all *Cryptosporidium* strains and species has been shown to be $\leq$ 5 oocysts. Therefore, the investigators focused on the role of the immune system in disease expression and parasite clearance (as opposed to infection) in gnotobiotic pigs in this set of experiments. Immunosuppression of pigs was achieved through daily oral dosing with the steroids dexamethazone (dex) from 1 to6 days of age, followed by prednisolone (pred) from 1 to 4 weeks of age. Onset to oocyst shedding from infection was found to be shorter in the dex/pred treated versus untreated Genotype 2 (GCH1)-inoculated pigs and the duration of oocyst shedding (or patent period) longer. However, no difference in severity of clinical disease (diarrhea) was observed between dex/pred treated and untreated Genotype 2-inoculated pigs.

Cellular Immunity: Cytokine Responses: The investigators evaluated the correlation between levels of several intestinal cytokines and *C. parvum* pathogenesis in 1 day-old gnotobiotic pigs given approximately 5×10^6 Genotype 2 (GCH1 or OH) *C. parvum* oocysts. A significant correlation between time, tissue, and cytokine messenger RNA (mRNA) expression was observed, which appeared dependent on parasite presence or absence and not severity of tissue pathology. Because of the significant differences observed between the pathogenesis of Genotypes 1 and 2 of *C. parvum* in gnotobiotic pigs (summarized previously), another assay was developed to quantitate expression of cytokine mRNA in the intestinal tissues of 1 day-old pigs inoculated with a low dose Genotype 1 or 2 *C. parvum*. Unlike the previous cytokine study, no significant correlation was found between time, tissue, and cytokine mRNA level, and no significant differences in cytokine mRNA expression were found between the Genotype 2-infected and Genotype 1-infected pigs. These studies suggest that *C. parvum* infection, regardless of infecting strain or its virulence, elicits mucosal cytokine responses in gnotobiotic pigs that significantly contribute to parasite clearance and recovery.

Humoral Immunity: Serum Immunoglobulin (Ig) Responses: In this series of experiments, the investigators examined and compared the humoral immune responses of neonatal gnotobiotic pigs inoculated with high doses of parental Genotype 2 (GCH1 and OH) *C. parvum* isolates. Twenty-three percent of pigs were found to have developed *Cryptosporidium*-specific IgM serum antibodies, while only 2 percent developed IgG and IgA serum antibodies at 6-10 days post-inoculation (PI). A second assay was utilized to compare the serum antibody responses of gnotobiotic pigs that were: (1) *C. parvum*-infected and dex/pred-treated (immunosuppressed) versus untreated; (2) *C. parvum*-infected with high

(> 10,000 oocysts) versus low (< 10 oocysts) inoculating dose; and (3) *C. parvum*-infected with Genotype 1 (2 strains) versus Genotype 2 (GCH1 and OH) strains. First, all of the non-immune-suppressed *C. parvum*-infected pigs developed *Cryptosporidium*-specific serum antibodies by 3 weeks post infection (PI), whereas 57 percent of the dex/pred-treated pigs failed to develop *Cryptosporidium*-specific serum IgG, and 29 percent failed to develop detectable *Cryptosporidium*-specific serum IgM or IgA by 5 weeks PI. Second, the dose of *C. parvum* used to infect the pigs was found to influence the subsequent antibody responses with low doses of Genotype 2 generally eliciting a slower and lower serum IgG response and a slower but higher serum IgM and IgA response, compared to high doses. High doses of Genotype 1 and Genotype 2 *C. parvum* isolates or low doses of Genotype 2 isolates elicited serum antibody responses in all (100 percent) pigs tested, whereas low doses of HuG1 *C. parvum* did not (~20 percent did not respond). Finally, the *C. parvum* genotype was found to influence the antibody response, with Genotype 1 *C. parvum* eliciting lower IgG, IgM, and IgA responses than low doses of Genotype 2 *C. parvum*.

Summary: This research project has successfully used a neonatal gnotobiotic pig model to derive single oocyst progeny (clones) of Genotypes 1 and 2 C. *parvum* isolates and to assess risk factors for cryptosporidiosis. Infection studies using the Genotype 2 (GCH1 and OH) clones clearly show them to be more virulent than the parental strains, while variability in the clinical responses of pigs to different pools of each C. *parvum* clone was greatly reduced compared to parental isolates. This suggests that the cloning procedure effectively derived a genetically homogenous (clonal) population of parasites compared to the parental strains. The availability of both Genotype 1 and 2 *C. parvum* clones for viability, infectivity, genetic, and immunologic studies constitutes a significant advancement in *Cryptosporidium* research. This research also supports a hypothesis that *Cryptosporidium* "virulence" is determined primarily by host species and parasite species-level variations, and only minimally by differences among strain within a species. Lastly, these studies provide biological evidence to support previously published genomic studies that suggest Genotype 2 and 1 C. *parvum* isolates are separate and distinct species of *Cryptosporidium* (*C. parvum* and *C. hominis*, respectively).

Investigator

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For More Information

NCER Project Abstract and Reports:

http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/191/report/0

Peer Reviewed Publications

Nielsen, C.K., and Ward, L.A. 1999. Enhanced detection of *Cryptosporidium* in the acid-fast stain. Journal of Veterinary Diagnostic Investigations 11:567-569.

Ward, L.A., and Wang, Y. 2001. Rapid methods to isolate *Cryptosporidium* DNA from frozen feces for PCR. Diagnostic Microbiology and Infectious Diseases 41:37-42.

Morgan-Ryan, U.M., Fall, A., Ward, L.A., Sulaiman, I., Fayer, R., Andrew-Thompson, R.C., Olson, M., Lal, A., and Xiao, L. 2002. *Cryptosporidium hominis* n.sp. (Apicomplexa: Cryptosporidiidae) from *Homo sapiens*. Journal of Eukaryotic Microbiology 49:433-440.

Pereira, S.J., Ramirez, N.E., Xiao, L., and Ward, L.A. 2002. Pathogenesis of human and bovine *Cryptosporidium parvum* in gnotobiotic pigs. Journal of Infectious Diseases. 186(5):715-718.

Sestak, K, Ward, L.A., Sheoran, A., Feng, X., Akiyoshi, D.E., Ward, H.D., and Tzipori, S. 2002. Variability among *Cryptosporidium parvum* Genotype 1 and 2 immunodominant surface glycoproteins. Parasite Immunology 24:213-219.

Foster, J.C., Glass, M.D., Courtney, P.D., and Ward, L.A. 2003. Effect of *Lactobacillus* and *Bifidobacterium* on *Cryptosporidium parvum* oocyst viability. Food Microbiology 20(3):351-357.

Glass, M.D., Courtney, P.D., LeJeune, J.T., and Ward, L.A. 2004. Effects of *Lactobacillus acidophilus* and *L. reuteri* cell-free supernatants on *Cryptosporidium* viability and infectivity *In Vitro.* Food Microbiology 21:423-429.

Ramirez, N.E., Xiao, L., Ward, L.A., and Sreevatsan, S. 2005. Derivation of *Cryptosporidium hominis* progeny in the gnotobiotic pig model. Parasitology (accepted).