The Epidemiology of *Cryptosporidium* Infection: Analyses of the Roles of Drinking Water and Wildlife

Joseph F. Perz

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ABSTRACT

The prevention of *Cryptosporidium* infection is complicated by the lack of knowledge regarding its transmission routes. This dissertation was aimed at addressing this shortcoming using dual approaches: (1) risk assessment and modeling; and (2) molecular epidemiology. A framework was developed to examine the potential role of tap water in the transmission of endemic *C. parvum* infection. The resulting model incorporated uncertainty analysis and had two main components: exposure-infection, to relate low dose exposure to infection; and infection-outcome, to incorporate probabilities of clinical outcomes leading to case detection and reporting. The population was divided into four subgroups: adults and children with and without AIDS. The model output was consistent with the premise that *Cryptosporidium* infection is substantially under-reported in the general population and the fact that the majority of cases are detected in persons with AIDS. Further, it was shown that this pattern was consistent with a common source exposure; low-level transmission via drinking water may represent an important and unrecognized transmission route. The need for improved investigations of *Cryptosporidium* epidemiology was addressed through the development of techniques for the detection and characterization of *Cryptosporidium* in fecal samples. Examination of the *Cryptosporidium parvum* rDNA unit from a typical human isolate demonstrated the presence of two distinct units, termed Types A and B, as had been previously shown for the *Cryptosporidium parvum* bovine genotype. This work provided a foundation for speciation and genotyping of *Cryptosporidium* isolates on the basis of DNA sequence analysis. These techniques were applied to a survey of mammalian wildlife in lower New York State. Evidence for a sylvatic transmission cycle for *C. parvum* involving white-tailed deer and a variety of rodents and other hosts was found. This cycle appears to involve the bovine genotype, previously shown to occur in approximately 20% of human cases. In addition, at least one novel *C. parvum* strain was detected, involving at least three different host species. These findings require further investigation, but recent reports of human infections with novel *Cryptosporidium* strains indicates their relevance to public health.

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I. INTRODUCTION

The emergence of *Cryptosporidium* and cryptosporidiosis has challenged public health on numerous levels, often in profound ways. *Cryptosporidium* has forced reassessments of traditional views and approaches in areas related to environmental protection, disease surveillance, medical treatment, food safety, law and economics. Despite the considerable research effort and significant progress made, many of the basic problems surrounding *Cryptosporidium* still lack solutions. In particular, the continued absence of an effective therapy means that prevention remains of paramount importance. And yet, prevention strategies are handicapped because the relative contributions of different sources and exposure routes remain unknown. The work described in this dissertation represents an attempt at increasing our understanding of the epidemiology of *Cryptosporidium* infection with the hope of contributing to the development of effective responses and interventions.

A. Background

The first cases of *Cryptosporidium* infection in humans were reported in 1976, but it was not until the early 1980's that the significance of this pathogen became known (Fayer *et al.* 1997). The advent of the acquired immunodeficiency syndrome (AIDS) epidemic revealed *Cryptosporidium* as an opportunistic pathogen capable of severely impacting human health. An intracellular protozoan parasite of vertebrate epithelia, *Cryptosporidium*, was first described in laboratory mice by Ernest Edward Tyzzer in

1907 (Fayer *et al.* 1997). The parasite only began to emerge from obscurity in the early 1970's when infections associated with economic losses in livestock were reported (O'Donoghue 1995). Since then, numerous reports of cryptosporidiosis (clinical *Cryptosporidium* infection) have been made in a wide range of hosts including mammals, birds, reptiles and fish (O'Donoghue 1995). Symptoms in mammals primarily involve watery diarrhea and infections are generally self-limiting. However, a broad spectrum of outcomes following infection has been documented ranging from asymptomatic infections to chronic life-threatening illness.

B. Public Health Significance

Cryptosporidiosis is an important enteric disease worldwide, both in developing and developed regions (Guerrant 1997). Significant morbidity occurs in the United States and other developed countries, most notably during outbreaks. For example, approximately 400,000 residents of Milwaukee experienced watery diarrhea during a waterborne cryptosporidiosis outbreak in 1993 (Mac Kenzie *et al.* 1994). Cryptosporidiosis is an opportunistic infection in persons with AIDS in whom the disease is profoundly debilitating and life threatening (Colford *et al.* 1996). It has been shown that 2-4 % of AIDS patients in developed countries had cryptosporidiosis at the time of AIDS diagnosis (Ungar 1990). Hospital-based studies have found 10-20% of AIDS patients with diarrhea to be infected with *Cryptosporidium* (Juranek 1995). Since there is no consistently effective treatment for cryptosporidiosis (Blagburn and Soave 1997), reducing the exposure of vulnerable populations to infection remains paramount.

Cryptosporidiosis is an important cause of persistent diarrhea in infants and children in the developing world (Guerrant 1997). *Cryptosporidium* infection in developing regions tends to be highly endemic. For example, prevalences of 32 and 40% were found in stool surveys of healthy children in Bolivia and healthy persons of all ages in southern India, respectively (Esteban *et al.* 1998; Kang *et al.* 1998). The endemic rates in developed countries, as measured by stool analysis (0.2% of non-diarrheal stools), are considerably lower (Guerrant 1997). However, developed regions are subject to occasional outbreaks during which large proportions of the exposed population can be infected (Guerrant 1997; Kramer *et al.* 1996; Mac Kenzie *et al.* 1994; Smith and Rose 1998). Extensive environmental contamination as well as person-to-person transmission are thought to contribute to the high endemic rates of *Cryptosporidium* infection seen in impoverished populations in developing countries (Newman *et al.* 1993; Zu *et al.* 1994). The relative contribution of oocyst sources and exposure routes to endemic *Cryptosporidium* infection are not known and may vary considerably in different regions and populations.

In developed regions, waterborne exposure has been well-documented in outbreaks of cryptosporidiosis (Kramer *et al.* 1996; Moore *et al.* 1993; Solo-Gabriele and Neumeister 1996). Oocysts are ubiquitous in surface water supplies worldwide (Newman *et al.* 1994; Rose *et al.* 1991; Smith and Rose 1998; Zuckerman *et al.* 1997) and they are routinely detected in drinking water (LeChevallier and Norton 1995). Two features of *C. parvum* biology are particularly pertinent in this regard. First, the high infectivity as evidenced by very low infectious doses (DuPont *et al.* 1995; Newman *et al.* 1993). Second, the oocysts are extremely resistant to degradation under a wide variety of conditions,

including chemical disinfection (Korich *et al.* 1990; Meinhardt *et al.* 1996). Epidemiologic evaluation of the contribution of tap water to low level (endemic) transmission has not been made, in part because of the inadequacy of stool examination to detect infection (Okhuysen *et al.* 1998; Weber *et al.* 1991), and because of the difficulties inherent in designing and implementing such studies (Centers for Disease Control and Prevention 1995).

Relative to common enteric pathogens, *Cryptosporidium* is significant in terms of the frequency with which it is isolated and its association with morbidity measures (Meinhardt *et al.* 1996). For example, in a large study conducted in England and Wales, *Cryptosporidium* was identified in laboratory stool submissions almost as often as *Salmonella* and more often than *Shigella*; among symptomatic children < 5 years old, *Cryptosporidium* was second only to *Campylobacter* in frequency (Public Health Laboratory Service Study Group 1990).

Cryptosporidium presents a particular challenge in both the developed and developing worlds. Even though exposure may be reduced by classic interventions for fecal-oral pathogens, such as the maintenance of adequate water supplies and sanitation (Esrey 1996), it cannot be eliminated. This is demonstrated by the high levels of exposure and infection inferred from seroprevalence surveys in developed countries, as well as the occurrence of waterborne outbreaks as discussed below. Surveys of limited U.S. population samples have found from approximately 15 to greater than 50 percent of subjects to be seropositive for antibodies to *Cryptosporidium* (DuPont *et al.* 1995; Kuhls

et al. 1994; Ungar 1990) suggesting that exposure and infection are common occurrences (Ungar 1990). Furthermore, considerable uncertainty surrounds the contribution of drinking water in the transmission of endemic disease (Juranek 1995). This uncertainty is a major obstacle in the formulation of strategies to reduce the risk of infection.

C. Transmission and Epidemiology

Transmission of *Cryptosporidium* occurs when the oocysts are excreted from an infected host and ingested by the next host. *C. parvum*, the species of primary importance to human health, has a broad host range and infects a wide range of mammals (Fayer *et al.* 1997). Transmission may occur directly from contact with an infected host (person-to-person or zoonotic) or indirectly, via drinking or recreational waters or from contamination of foods (Centers for Disease Control and Prevention 1996a; Meinhardt *et al.* 1996). Some studies have suggested that the contribution of sexual practices may be important among persons with HIV infection, but the majority of cases are left unexplained (Pedersen *et al.* 1996; Sorvillo *et al.* 1994). Although reactivation of latent infections due to declining CD4 counts might play a role in the appearance of cryptosporidiosis (Juranek 1995), waterborne outbreaks have indicated the significance of recent exposures in the acquisition and expression of cryptosporidiosis in persons with AIDS (Goldstein *et al.* 1996; Vakil *et al.* 1996).

D. Life Cycle

Cryptosporidium's life cycle is shown in Figure 1-1. The oocyst is shed in a fully infectious form and is the only stage that is found outside the host; the remainder of the cycle occurs within a single host. For *C. parvum*, four sporozoites are released during excystation. Each sporozite is capable of initiating infection, which commences with invasion of an epithelial cell. Successful invasion leads to maturation and asexual multiplication. The resulting merozoites, which are structurally similar to the sporozoites. proceed to infect other host cells. Thereafter, multiplication can occur either sexually or asexually. Sexual reproduction leads to the formation of oocysts, which are also believed to have a capacity for autoinfection. Typically, millions of oocysts may be shed each day by an infected host (O'Donoghue 1995).

E. Classification

The genus *Cryptosporidium* falls within the phylum Apicomplexa (Figure 1-2) and is related to protozoan parasites such as *Plasmodium* and *Toxoplasma* (O'Donoghue 1995). *Cryptosporidium* has been classified with other enteric coccidian parasites in the suborder Eimeriorina. The list of recognized *Cryptosporidium* species has been plastic; of the 21 species that have been named, only seven or so are currently considered valid (Fayer *et al.* 1997; O'Donoghue 1995). This consolidation followed the review of cross-transmission and morphological studies (O'Donoghue 1995). Two *Cryptosporidium* species, *muris* and *parvum*, are of primary importance in mammals. These species have been distinguished by differences in their sizes and sites of infection. *C. parvum* has

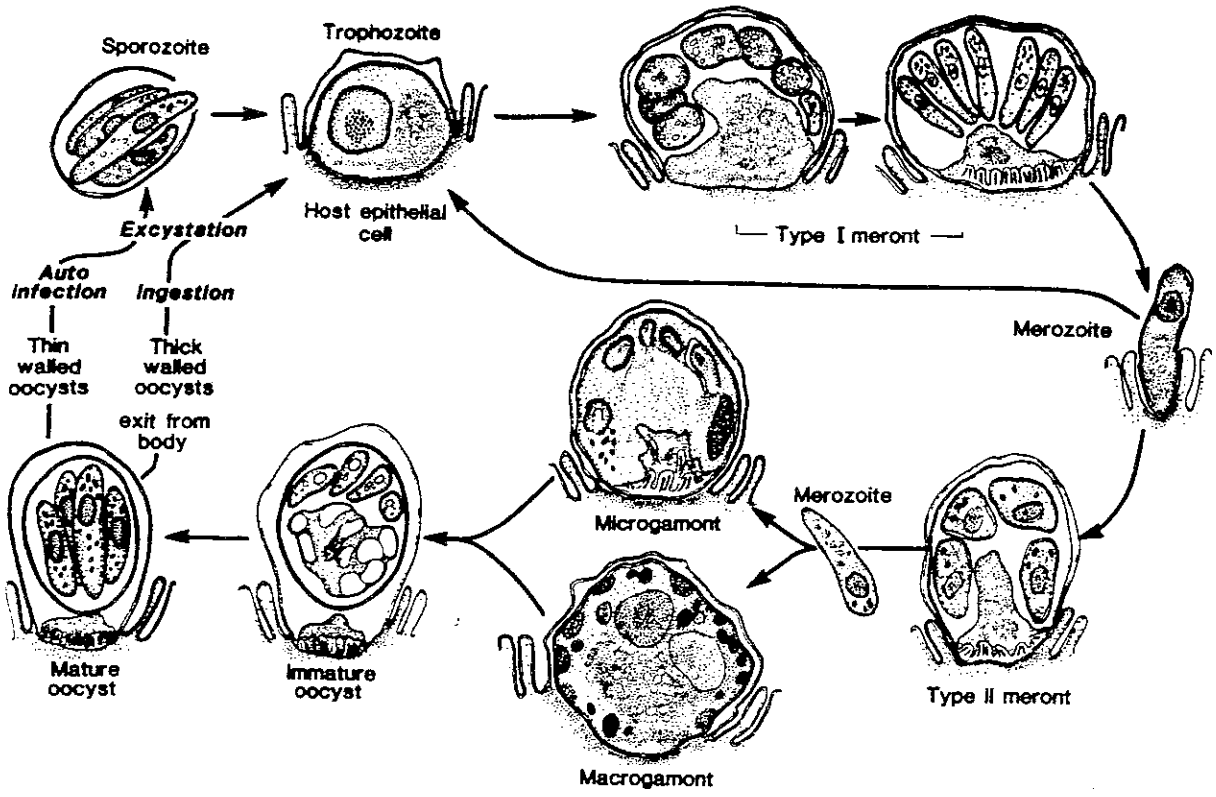


Figure 1-1: Diagrammatic representation of the life cycle of *C. parvum* (From Fayer, R., Speer, C.A., and Dubney, J.P., in *Cryptosporidiosis of Man and Animals*, Dubney, J.P., Speer, C.A., and Fayer, R., CRC Press, Boca Raton, FL, 1990)

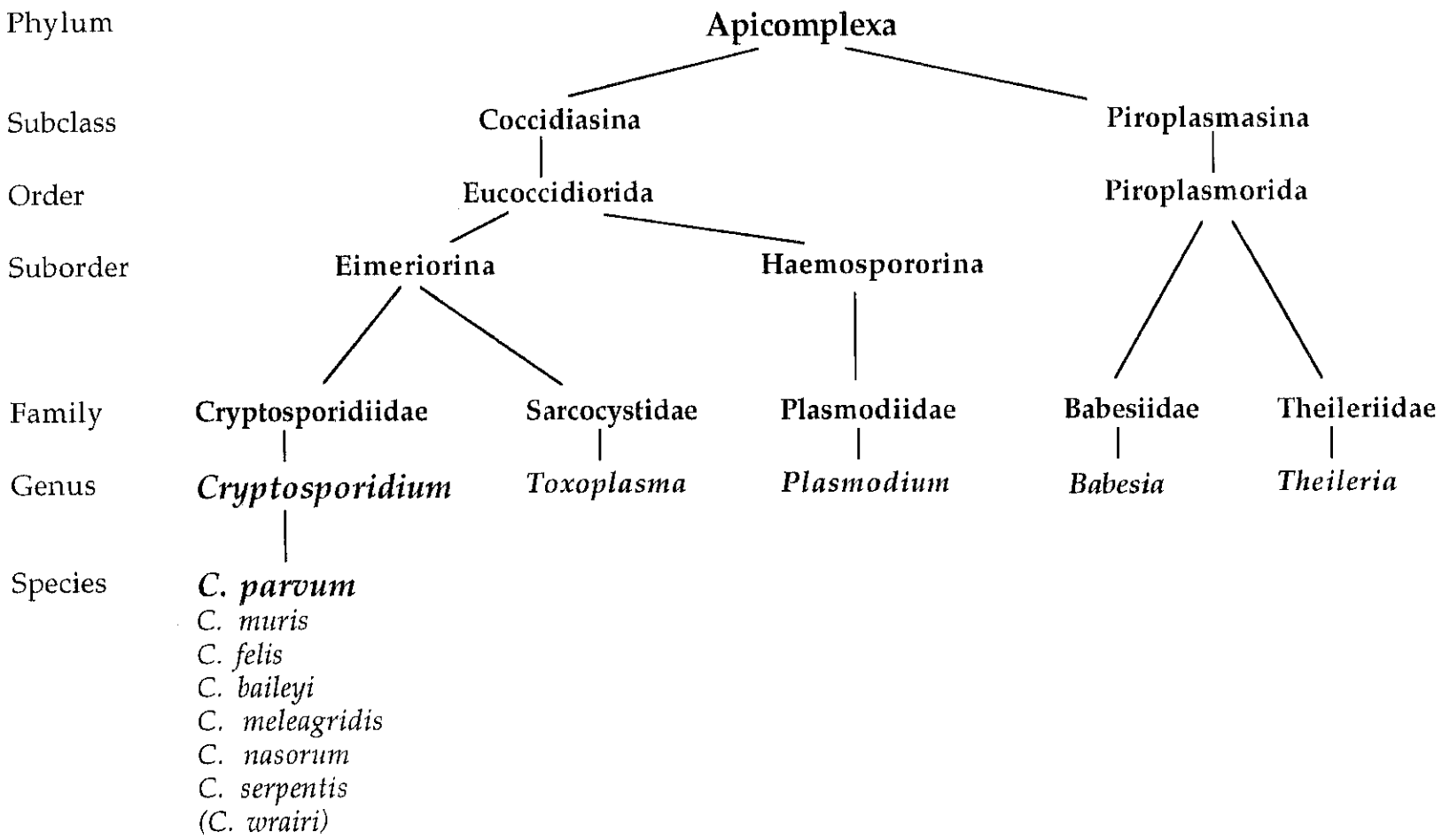


Figure 1-2: The Classification of *Cryptosporidium*

smaller oocysts (4-5 x 4-5 μm versus 7-8 x 5-6 μm for *C. muris*) and infects the small intestine rather than the stomach (O'Donoghue 1995). *C. muris* has been associated with a relatively small range of naturally infected hosts including only rodents (mice and rats) and hoofed mammals (e.g. cattle) (O'Donoghue 1995). In contrast, *C. parvum* infection has been reported in over 70 species of mammals belonging to nine orders (O'Donoghue 1995; Sturdee *et al.* 1999). The current status of two additional *Cryptosporidium* species infecting mammals remains unclear. These are *C. felis* and *C. wrairi*, isolated from cats and guinea pigs, respectively (O'Donoghue 1995). Based on newly available molecular and morphometric evidence, it appears that *C. felis* is valid (Bornay-Llinares *et al.* 1999; Sargent *et al.* 1998) whereas *C. wrairi* may not be (Xiao *et al.* 1998). Indeed, as the use of molecular tools to characterize *Cryptosporidium* isolates accelerates, the roster of recognized *Cryptosporidium* species will surely evolve. The current species classifications should be regarded as tentative (O'Donoghue 1995).

F. The Human and Bovine *C. parvum* Genotypes

Within *C. parvum*, two distinct genotypes have been described (Peng *et al.* 1997; Widmer 1998; Xiao *et al.* 1998). Both have a worldwide distribution and are associated with cryptosporidiosis. The "bovine" genotype has been isolated from humans and other mammals, notably livestock. In contrast, the "human" genotype has only been isolated from humans. Evidence for the two genotypes comes from a variety of molecular studies that found differences in antigen and isoenzyme profiles, restriction fragment length polymorphisms and DNA sequence heterogeneities (Widmer 1998). It is possible that

each genotype may circulate in distinct transmission cycles, one being anthroponotic and the other being zoonotic or a combination of both. Water may serve as a vehicle for both genotypes as confirmed by retrospective typing of isolates from outbreaks (Xiao *et al.* 1998). A recent review of genotyping studies showed that overall, 81 percent (118 out of 146) of isolates from human clinical samples were of the human genotype(Xiao *et al.* 1998).

G. *Cryptosporidium* Ecology

The natural ecology of *Cryptosporidium* probably involves a sylvatic cycle with transmission among wild vertebrates in which the infection may be largely asymptomatic (Current and Reese 1986). The high infection rates seen in domestic animals, particularly dairy cattle, indicate that a parallel zoonotic cycle occurs in agricultural settings (Meinhardt *et al.* 1996). An anthroponotic cycle also occurs either with direct person-to-person transmission, or indirectly as when human sewage contaminates the drinking water supply (Meinhardt *et al.* 1996). Water may serve as an important intersection for these cycles, since the effluent from sewage treatment plants and septic tanks and run off from agricultural lands and forests all enter the surface water supply (LeChevallier and Norton 1992; Ongerth and HH 1987; Smith and Rose 1990). Lending support to this premise is the observation that rainfall appears to have been an important factor in some drinking water outbreaks (Rose 1997). These cycles are likely to be intertwined in many more, often unexpected, ways. For example, transmission following exposures to livestock is well documented, and companion animals have also been implicated in

human infections (Juranek 1995; Meinhardt *et al.* 1996). Waterfowl have been shown to have the capacity to serve as carriers that may passively spread contamination from one environment to another (Graczyk *et al.* 1998). Likewise, filter-feeding shellfish such as oysters were shown to concentrate oocysts in their environment and thus present a risk to persons consuming them (Xiao *et al.* 1998).

H. The Role of Drinking Water

1. Outbreaks

Cryptosporidium is now regarded as a leading cause of waterborne outbreaks associated with drinking water. Since the first recognized waterborne *Cryptosporidium* outbreak in 1984 in Braun Station, Texas, more than a dozen outbreaks have occurred in North America alone, in both surface and ground water supplies (Rose 1997). In every outbreak, *Cryptosporidium* was detected first in the exposed and infected population before the water supply was suspected or implicated (Meinhardt *et al.* 1996).

Interestingly, while unfiltered surface water supplies have experienced outbreaks, most major incidents were associated with filtered systems; this observation has led to the hypothesis that acquired immunity at the community level may play a role in outbreaks (Frost *et al.* 1997). Outbreaks have often occurred in the absence of identified treatment irregularities or violations of regulatory standards (Meinhardt *et al.* 1996). For these

reasons, *Cryptosporidium* has forced reassessments of standard approaches to the supply and regulation of drinking water, as discussed below.

2. Endemic Transmission

The role of tap water in the transmission of microbial illness exclusive of outbreaks is unclear. Substantial concern persists that low levels of pathogen occurrence may be responsible for the transmission of background (endemic) levels of enteric disease (Macler and Regli 1993; U.S. Environmental Protection Agency, 1994). For example, by conducting randomized controlled interventions in which Canadian households were provided either real or sham tap water filters, Payment *et al* showed 35% and 14-19% reductions in the incidence of gastrointestinal illness, in two separate trials (Payment *et al.* 1991; Payment *et al.* 1997).

Cryptosporidium is a prime candidate for worrisome levels of endemic transmission, because it is ubiquitous in surface waters and is extremely resistant to various environmental pressures, including chemical disinfection; few, if any, barriers to its passage exist in water supplies, and none of the barriers, including filtration, can be considered fail-safe (Centers for Disease Control and Prevention 1995; Meinhardt *et al.* 1996). It is clear that transmission of *C. parvum* from water supplies may frequently go unrecognized (Frost *et al.* 1996; Goldstein *et al.* 1996; Juranek 1995). Besides large-scale outbreaks of the type that occurred in Milwaukee in 1993 (Mac Kenzie *et al.* 1994), more limited waterborne outbreaks have been documented; the distinction between

epidemic and endemic transmission is obscured by the limited capacity to recognize an outbreak (Frost *et al.* 1996). For example, the 1994 Clark County, Nevada (Las Vegas) outbreak could easily have escaped detection had a sizable AIDS population and active surveillance system for cryptosporidiosis both not been present (Goldstein *et al.* 1996). A case-control study strongly implicated the water supply in this outbreak, despite its high quality and state-of-the-art treatment. It is also notable that the limited monitoring of the water supply did not detect any *Cryptosporidium* oocysts (Goldstein *et al.* 1996; Roefer *et al.* 1996). Similarly, a case-control investigation of a small cryptosporidiosis outbreak in 1991 in South London demonstrated a significant association with tap water ingestion, including a dose-response effect (Maguire *et al.* 1995). Detection of this outbreak was attributed to the affected population having been served by a regional public health laboratory that had the unusual practice of screening all stool samples for *Cryptosporidium*.

3. Regulatory Perspective

The response to the threat of waterborne *Cryptosporidium* from regulatory and public health authorities has evolved slowly. Disinfection has long been the primary means of making water safe from infectious disease risks, with filtration an integral but secondary barrier (Rose 1997). More so than *Giardia* or any waterborne pathogen yet described, *Cryptosporidium* has the ability to circumvent this strategy (Meinhardt *et al.* 1996; Rose 1997) – the secondary barrier is not entirely effective and the primary barrier is essentially absent. In addition, difficulties have been encountered in monitoring

Cryptosporidium's presence in water, in developing new and affordable treatment technologies, and in characterizing the risks of outbreak and endemic disease transmission (Centers for Disease Control and Prevention 1995; Rose 1997). The US Environmental Protection Agency began development of regulations to address *Cryptosporidium* in 1994 in its proposed Enhanced Surface Water Treatment Rule (ESWTR) (U.S. Environmental Protection Agency, 1994). An interim version of this rule was recently promulgated, to take effect by December 2001 for large surface water systems (those serving more than 10,000 persons) (U.S. Environmental Protection Agency, 1998). This treatment optimization rule essentially formalizes the strategy that has evolved among water utilities. The rule requires filtered systems to provide 2-log (99%) removal of *Cryptosporidium* spp. and unfiltered systems to pursue aggressive *Cryptosporidium* controls in their watersheds. In addition, turbidity, which may serve as a surrogate indicator for *Cryptosporidium*, will be more tightly limited and monitored.

I. Objectives

In order to further the understanding of the epidemiology of *Cryptosporidium* infection, in support of the development of effective strategies for protecting public health, this dissertation was structured around two objectives as follows:

> Objective #1 – To develop the principles of risk assessment and mathematical modeling for application to the epidemiology of *Cryptosporidium* infection, with special attention to environmental exposure via tap water.

> Objective #2 – To develop and apply the tools of molecular biology in the investigation of the epidemiology of *Cryptosporidium* infection in humans and sylvatic mammals, with special attention to its detection, genotypic differentiation, and environmental distribution.

The following chapters, II and III, correspond to Objective # 1 and #2, respectively, and present the related rationales, specific aims and studies.

II. CRYPTOSPORIDIUM IN TAP WATER: RISK ASSESSMENT AND MODELING APPROACHES

A. Introduction

While waterborne transmission is well established in community outbreaks of cryptosporidiosis, the significance of low levels of occurrence of this parasite in non-outbreak settings remains unclear. *Cryptosporidium* is ubiquitous in the environment and is highly resistant to standard water disinfection practices (Centers for Disease Control and Prevention 1995). Therefore, the occasional presence of this pathogen in tap water may be responsible for the transmission of background (endemic) levels of enteric disease (Payment *et al.* 1997; U.S. Environmental Protection Agency, 1994). Epidemiologic analyses of the contribution of tap water to *Cryptosporidium* infection in the endemic setting are not yet available. Therefore, a risk assessment approach - i.e. an approach predicting endemic disease rates as a function of the concentration of *Cryptosporidium* in drinking water supplies - may contribute to our understanding of the significance of the water route and to the formulation of strategies for the protection of public health (ILSI Risk Science Institute Pathogen Risk Assessment Working Group 1996).

Dose-response data for *Cryptosporidium* from an experiment involving healthy human volunteers were published in 1995 (DuPont *et al.* 1995). These data provide information that may be used in a risk assessment model relating exposure via tap water to infection

risks. Methods for the risk assessment of infectious microorganisms are not well established. Therefore, the development of an appropriate framework for a risk assessment of *Cryptosporidium* in tap water was required before a population-based case study could be performed that would examine this issue in detail. The following specific aims were formulated in order to address the outlined goals:

Specific Aim #1A: To develop a framework that allows the estimation of theoretical infection risks in populations exposed to *Cryptosporidium* in tap water, and the comparison of these risks with occurrence data derived from case reports.

Specific Aim #1B: To extend the framework developed in Specific Aim 1A by incorporating quantitative uncertainty analysis, and to examine the potential role of tap water in the transmission of endemic *Cryptosporidium* infection, comparing model predictions to observed rates of cryptosporidiosis in New York City in 1995.

The remainder of this chapter is organized into two sections, which follow below. Section B relates to Specific Aim #1A while section C relates to Specific Aims #1B.

B. Framework for Assessing the Risks of *Cryptosporidium* in Tap Water

Risk assessment has evolved into an integral component in the development of environmental policies and standards (Macler and Regli 1993; National Research Council 1983). In essence, risk assessment applies the best available scientific evidence to address questions or problems for which there may be no scientifically valid or definitive answer. Quantitative risk assessment originated in the analysis of nuclear/radioactive hazards, and has been used extensively in relation to chemical/toxic hazards. The application of risk assessment to microbial hazards is relatively recent, but the basic components are held in common. These components are hazard identification, exposure assessment, dose response assessment and risk characterization (National Research Council 1983).

In developing a framework for examining waterborne *Cryptosporidium* it was necessary to define appropriate endpoints and goals. The goal here was to examine whether available information on the infectivity and the occurrence of *Cryptosporidium* in drinking water were compatible with available information regarding the rates and distribution of cases. This required a population-based approach. Also, the use of conservative assumptions, integral to chemical risk assessment, was not compatible with the stated goal. Practically, this meant not incorporating safety factors and the use of best estimates of central tendency for all parameters in the model. (Note that even if the goal of the present analysis had been regulatory rather than epidemiologic, the avoidance of "worst case scenarios" and excess conservatism would still be important. This is because

reductions in microbial risk may be accompanied by a rise in chemical risk, as from increased exposure to disinfectants and disinfectant by-products (Macler and Regli 1993; Putnam and Graham 1993). Another key difference with chemical risk assessments concerns the definition of appropriate endpoints (Macler and Regli 1993; Putnam and Graham 1993). Attention to the effects of chemical exposures is often focussed on chronic, narrowly-defined effects, such as cancer. On the other hand, the risk of waterborne microbial illness is associated with acute effects that may vary tremendously in their health effects, as is clearly the case for *Cryptosporidium*.

Dose response data from the human volunteer studies provided a means of relating exposure to the risk of infection (DuPont *et al.* 1995; Haas *et al.* 1996). However, direct comparison of risk assessment results with surveillance data for cryptosporidiosis cases is inappropriate because *Cryptosporidium* infections often go unreported (Centers for Disease Control and Prevention 1995). It is therefore important to consider the spectrum of clinical responses that is associated with infection with these organisms, and to relate these to the probability of case reporting. A framework was formally developed to incorporate the goals and assumptions described above (Perz *et al.* 1997). This framework was evaluated in a preliminary application (shown in Appendix A to avoid redundancy with the advanced model described below).

Briefly, the approach developed was as follows. Because the degree of hazard associated with *Cryptosporidium* infection varies by immune status, a separate analysis was made for persons with AIDS, in whom this infection tends to be persistent and profoundly

debilitating (Juranek 1995; Meinhardt *et al.* 1996). (This distinction also facilitates the relation of infection risk to the likelihood of case reporting, as discussed below.) Exposure was estimated from tap water intake data and from two assumed levels of *C. parvum* occurrence in tap water. To relate the exposure estimates to an annual risk of infection, the results of a published dose-response analysis based on the volunteer study were adopted (Haas *et al.* 1996).

A source of data on infection rates is provided by epidemiologic surveillance programs, which generally consist of the facilitated reporting of infections from laboratories that perform stool examinations. Typically, testing for *Cryptosporidium* is done only at the request of physicians, with the result that most requests come from clinicians who treat AIDS patients and who are aware of the disease (Boyce *et al.* 1996). In contrast, it has been demonstrated in outbreak situations that infections occurring in the general population, even when associated with medical attention, are unlikely to receive a confirmed diagnosis via stool examination for *Cryptosporidium* (Mac Kenzie *et al.* 1994). A sequence of events was derived in order to relate infection to illness severity, medical care utilization, diagnostic testing and case reporting. Quantitative estimates of each of the component events for the AIDS and non-AIDS subgroups were derived and applied to the infection risk estimates. Results were presented in the form of projected case rates for populations with varying prevalences of AIDS. Broadly, these results appeared to be reconcilable with information available from surveillance programs (Perz *et al.* 1997).

C. Comparison of Predicted Risks with Observed Levels of Disease

1. INTRODUCTION

The framework developed under the previous specific aim provided a useful approach for relating environmental exposure to *Cryptosporidium*, via tap water, to infection risks and available disease surveillance information (Perz *et al.* 1997). Application of the framework to a population-based case study, however, dictated that certain refinements be incorporated. For example, the utility of a model is increased when its inherent uncertainty is estimated and presented in quantitative terms (Roseberry and Burmaster 1992). This provides a sense of the overall range in the estimates and the identification of those model components or parameters that contribute most to the overall uncertainty. It was also determined that the model should consider children separately, because they may be more severely affected by infection and/or more likely to be diagnosed and reported (LeChevallier *et al.* 1991).

Compared to Specific Aim 1A, which focused on establishing the framework for this modeling approach, this specific aim attempted a more rigorous examination of the potential role of tap water in the transmission of endemic *C. parvum* infection. Because endemic waterborne transmission appeared plausible, a more in-depth evaluation of such a role was considered important. New York City provided a useful setting for this application of the model because it has active surveillance for cases of cryptosporidiosis

(Miller *et al.* 1997), a large population, a high prevalence of AIDS, and a surface water supply which may represent a source of low level exposure to *C. parvum*.

As noted in the previous section, the spectrum of clinical responses associated with *C. parvum* infection poses considerable difficulties in the study of this pathogen. A striking observation was made by Mac Kenzie *et al.* in their investigation of the 1993 Milwaukee outbreak: "(our) findings suggest that people with diarrhea seek health care infrequently, do so only when the illness is severe or prolonged, and are unlikely to be tested for *Cryptosporidium* infection" (Mac Kenzie *et al.* 1994, p. 166). In order to relate risk assessment estimates of endemic waterborne *C. parvum* infection with the case counts available from surveillance efforts, the probabilities embedded in this statement have been quantified, as outlined below.

2. MATERIALS AND METHODS

a. Overview of the risk assessment approach used in this study

Figure 2-1 outlines the framework used to address the question: what numbers of infections and detected cases are expected to occur in a population exposed to a low concentration of infective *Cryptosporidium* oocysts via its tap water? Given a long term average concentration, the estimated numbers of infections due to ingestion of tap water will depend upon parameters which characterize host-microbe interactions in the exposed population. The total population is divided into four main subgroups, as described below.

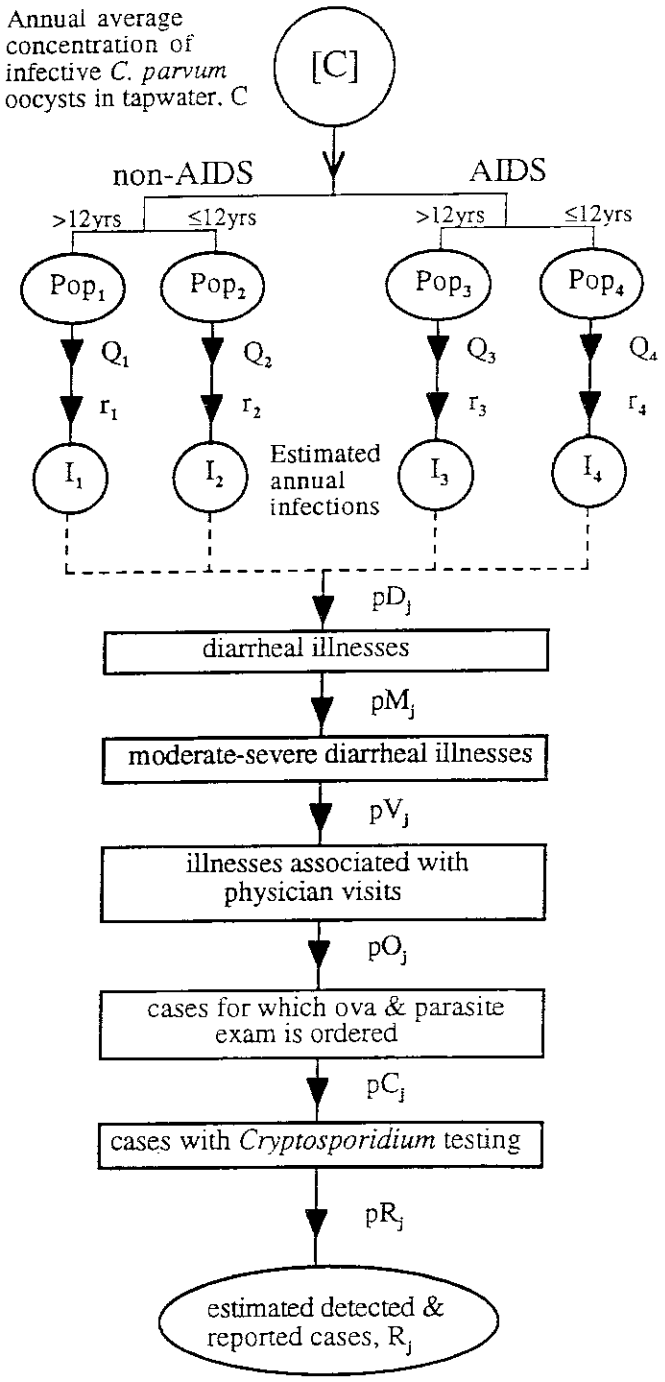


Figure 2-1: Conceptual framework for predicting the numbers of *C. parvum* infections and other outcomes leading to case reports to a Health Authority. The population is divided into four subgroups: non-AIDS > 12 yrs; non-AIDS ≤ 12 yrs; AIDS > 12 yrs; and AIDS ≤ 12 yrs, designated 1, 2, 3, and 4, respectively. The conditional probabilities (pN_j) are determined for each subgroup (j). Pop = number of persons in each subgroup; Q = average annual tapwater intake; r = infectivity (dose response parameter); I = estimated annual infections; pN_j = conditional probabilities (see text for full details).

For each subgroup, the annual intake of tap water governs the total exposure occurring by this route. Because we are evaluating low level exposure, we utilize estimates of the probability that exposure to a single oocyst will lead to a clinical infection (i.e., a dose of one is considered potentially infective). Combining the exposure and dose-response data provides an estimate of the likelihood and number of infections for each subgroup. The probability of enteric illness including diarrheal symptoms may in turn be estimated. Risk assessments usually stop at or before this point, because it is difficult to go further on the basis of the dose-response data. However, we proceed to derive estimates of additional outcomes leading to the detection and reporting of a case.

The mathematical model derived from the framework has two major endpoints: the numbers of infections and numbers of reported cases for each subgroup. The model presented here is basically multiplicative in nature, except that the total numbers of infections and cases were obtained by summing across the subgroups. The percentage of infections and cases occurring in the subgroups were calculated and compared to an observed distribution.

Uncertainties in the model were assessed by incorporating ranges and distributions for the input parameters followed by determination of the uncertainties in the model outputs. Appropriate inputs for each parameter are central tendency estimates for the subgroup in question, along with 95 percent confidence intervals encompassing both uncertainty in the estimate and variability in the measure. A hybrid method, chosen for its relative transparency, was used: direct calculations for the multiplicative components with Monte

Carlo analysis, i.e., an analysis of the distribution of predicted results, based on the uncertainties of the input parameters, for the overall results (Eisenberg *et al.* 1996; Slob 1994).

b. Exposure-Infection Model

The model for relating low dose exposure to infection is:

$$I_j = C \times Pop_j \times Q_j \times r_j$$

where:

I	=	calculated number of infections per year
C	=	relevant *C. parvum* concentration (organisms/L)
Pop	=	population of exposed subgroup (persons)
j	=	subgroup
Q	=	annual tap water intake (L/yr)
r	=	single organism infectivity (infection/organism/person)

Concentration assessment. For the purposes of this risk assessment, an estimate of the long term (e.g., annual) average occurrence in tap water of viable *C. parvum* oocysts which are both infective and pathogenic to humans is required; in other words, the true concentration that is relevant to public health. This concentration has not been reliably measured to date because of shortcomings in the testing method in general use (fluorescent antibody staining and microscopic analysis of a portion of large volume filtered water samples (Centers for Disease Control and Prevention 1995)). The present analysis evaluated a plausible baseline, or unit, concentration of one oocyst per thousand liters, because of the large degree of uncertainty associated with this parameter.

Measurements reported for drinking water from surface supplies in the U.S. (LeChevallier and Norton 1995; LeChevallier *et al.* 1991; New York City Department of Environmental Protection 1997; Rose *et al.* 1991) suggest average concentrations of approximately one *Cryptosporidium* oocysts per 100 or 1,000 liters. A number of problems are associated with relating measured concentrations to a central tendency estimate of the true *C. parvum* concentration in a given water supply; these problems include: a) recovery efficiencies which are low (< 5-10 percent) and highly variable, b) a lack of information concerning viability, c) imperfect specificity of the antibody for *C. parvum* and d) detection limits of greater than one oocyst per 100 liters (Centers for Disease Control and Prevention 1995). Taken together these factors contribute substantial uncertainty to the central tendency estimate, and are characterized by a greater than 100-fold range in the 95 percent confidence interval (see Appendix B). Note that uncertainty in the central tendency estimate might be even greater if the stochastic distribution of oocysts in the environment and the timing, number, and size of the samples were fully considered.

Populations at risk of infection. A rationale for subdividing the exposed population is suggested from consideration of the Las Vegas outbreak (Goldstein *et al.* 1996). In that waterborne outbreak, most of the detected cases of cryptosporidiosis were identified in persons with AIDS, with the remainder primarily occurring in immunocompetent children. This pattern seemed to reflect: a) the strong tendency of persons with AIDS to experience severe outcomes following infection and awareness of and testing for the disease among their doctors (Goldstein *et al.* 1996), and, presumably, b) among persons

without AIDS, a tendency for children to be more severely affected and/or more likely to see a doctor than adults. Combining the two classifications results in four subgroups: adult AIDS, pediatric AIDS, adult non-AIDS and pediatric non-AIDS. The pediatric groups consist of children less than 13 years of age, consistent with the AIDS surveillance definition (Centers for Disease Control and Prevention 1992).

Population data for the non-AIDS segments were taken from the 1990 census, while the 1995 adult and pediatric AIDS populations were estimated from surveillance data collected by the New York City Department of Health (New York City Department of Health 1996) with adjustments to account for lags in reporting of diagnoses and incomplete reporting of AIDS deaths. Values used in the model are listed in Table 2-1.

Tap water intake rates. Available data on U.S. tap water intakes (Roseberry and Burmaster 1992) were adjusted to account for the fraction used in the preparation of hot foods and beverages (Pennington 1983). Because there is evidence that persons with AIDS may exhibit significant avoidance of tap water (Goldstein *et al.* 1996), consistent with published advice from AIDS advocacy organizations, their average intake was estimated to be further reduced relative to the non-AIDS population (Table 2-1).

Dose-response assessment, risk of infection. Dupont *et al.* conducted an infectivity experiment with healthy adult volunteers without serological evidence of prior exposure

Table 2-1: Inputs to the exposure-infection component of the model

		non-AIDS Adult			non-AIDS Pediatric		
parameter*	units	central tendency	95% confidence interval	dispersion factor, k†	central tendency	95% confidence interval	dispersion factor, k
population	1,000 persons	6080	5530 - 6690	1.1	1240	1130 - 1360	1.1
tapwater intake	liters/year	214	107 - 427	2	181	90 - 361	2
infectivity	infection/oocyst/person	0.0042	0.0017 - 0.0105	2.5	0.0042	0.0017 - 0.0105	2.5
		AIDS Adult			AIDS Pediatric		
parameter	units	central tendency	95% confidence interval	dispersion factor, k	central tendency	95% confidence interval	dispersion factor, k
population	1,000 persons	30	23 - 39	1.3	0.8	0.5 - 1.2	1.5
tapwater intake, baseline	liters/year	214	107 - 427	2	181	90 - 361	2
tapwater avoidance		0.3	0.1 - 0.9	3	0.3	0.1 - 0.9	3
tapwater, adjusted	liters/year	150	41 - 548	3.7	126	34 - 463	3.7
infectivity, baseline	infection/oocyst/person	0.0042	0.0017 - 0.0105	2.5	0.0042	0.0017 - 0.0105	2.5
infectivity multiplier		3	1 - 9	3	3	1 - 9	3
infectivity, adjusted	infection/oocyst/person	0.0126	0.0030 - 0.0526	4.2	0.0126	0.0030 - 0.0526	4.2

* Lognormal distributions are assumed for each parameter

† For a lognormally distributed variable X (Slob, 1994),
- the standard deviation, σ, is related to the dispersion factor, k, by the expression: $\sigma = \ln(k)/1.96$;
- k indicates deviation about the median, M, as $\text{Prob}\{M/k < X < kM\} = 0.95$;
- the range of the 95% confidence interval can be characterized by the ratio of its upper and lower limits, or k^2 ; and
- for $Y = X_1X_2.../X_3...$, generally, $k_Y = \exp\{\sqrt{(\ln^2 k_{X1} + \ln^2 k_{X2} + \ln^2 k_{X3} + ...)}\}$

children than in adults, age-specific attack rates during the Milwaukee outbreak were actually lower in the youngest age classes (Mac Kenzie *et al.* 1994); this could reflect similar susceptibility to infection, but lower tap water consumption.

Because the detection of an infection in the volunteer study depended on stool analysis, which requires relatively high densities of the parasite (approximately 5,000 oocysts per gram of feces (Weber *et al.* 1991)), it is likely that not all colonizations were detected in the healthy adults. Since persons with AIDS tend not to clear the parasite while severe immunosuppression is present, colonization may be more likely to be followed by infection and illness (Benhamou *et al.* 1995). Support for an up to 10-fold greater infectivity among persons with AIDS comes from calculations based on the Las Vegas outbreak utilizing approximate values for the detection limit of water monitoring, the duration of the outbreak, and disease rates among persons with and without AIDS (Goldstein *et al.* 1996; Roefer *et al.* 1996) (calculations not shown). Cryptosporidiosis in persons with HIV infection is manifested chiefly as a late stage infection, mainly evident in advanced AIDS (i.e., CD4+ T lymphocyte (CD4) counts < 100 cells/μl) (Colford *et al.* 1996; Goldstein *et al.* 1996; Hashmey *et al.* 1997). Since the infectivity is likely to increase as the CD4 count of the host declines, from perhaps the general population risk at CD4 counts above 200 to a five- or ten-fold higher infectivity at counts below 100 or 50, we use an approximate central tendency value of three-fold higher infectivity (0.0126) for persons with AIDS. Values are summarized in Table 2-1.

c. The infection-outcome model

Since definite diagnosis of *C. parvum* infection requires stool testing, only those infections resulting in illness and physician contact can come to the attention of the appropriate health agency. Testing for *Cryptosporidium* is done primarily at the request of physicians (Boyce *et al.* 1996), with the result that most requests come from physicians who are treating patients with AIDS (Goldstein *et al.* 1996; Morin *et al.* 1997). Diagnostic evaluation of patients with acute enteric illness is not always indicated because most cases resolve spontaneously, requiring, at most, supportive therapy (Park and Giannella 1993). Ova and parasite (O&P) testing is recommended when warranted by clinical suspicion and/or when the illness is prolonged, unresponsive, or medically important (e.g., immunocompromised host or a case with severe volume depletion, bleeding, high fever, etc.) (Guerrant and Bobak 1991; Park and Giannella 1993).

The sequence of events leading from infection to case reporting has been described (Frost *et al.* 1996). We modified and refined this sequence for *Cryptosporidium* infection, and cast these events as a series of conditional probabilities, in such a way that intermediate outcomes of interest could also be examined. The model that will be used to relate the estimated infections to the estimated number of reported cases is:

$$R_j = I_j \times p(R|I) = I_j \times pD_j \times pM_j \times pV_j \times pO_j \times pC_j \times pR_j$$

where:

R	=	calculated number of reported cases per year
I	=	calculated number of infections per year
j	=	subgroup
p(R|I)	=	p(case detection and reporting | infection)

pD = p(D | I) = p(diarrheal illness | infection)

pM = p(M | D) = p(moderate-severe illness | D)

pV = p(V | M) = p(physician visit | M)

pO = p(O | V) = p(ova & parasite exam | V)

pC = p(C | O) = p(*Cryptosporidium* test | O)

pR = p(R | C) = p(case detection and reporting | C)

Explanations of each of the component probabilities are presented in the following sections along with quantitative estimates and 95 percent confidence intervals (Slob 1994; Zar 1984) (summarized in Table 2-2).

p(diarrhea | infection), pD. Infection with *Cryptosporidium* may frequently be asymptomatic (Cordell and Addiss 1994; Current and Garcia 1991). The volunteer study provides an estimate that may be applicable to the general adult population. Though of a small sample size, this was the only study performed in an experimental setting. Of the 18 experimentally infected subjects, seven (39 percent) were completely asymptomatic, four (22 percent) had enteric symptoms without diarrhea, and seven (39 percent) had diarrhea with or without other symptoms (Chappell *et al.* 1996; DuPont *et al.* 1995); the development of symptoms was not correlated with the size of the dose (Haas *et al.* 1996). A probability of 0.7 (95 percent confidence interval (CI) 0.35-1.00) was estimated for the non-AIDS pediatric population, midway between the value of 0.4 (95 percent CI 0.20-0.80) applied to adults and the probability of 1.0 that might be expected for a first infection occurring in an infant. Reports of asymptomatic infections in persons with AIDS have been published, but it is not clear that those cases never experienced

Table 2-2: Summary of conditional probabilities used to relate *C. parvum* infection to case detection and reporting

conditional probability*	non-AIDS adult† central tendency estimate	non-AIDS adult† 95% confidence interval	non-AIDS pediatric central tendency estimate	non-AIDS pediatric 95% confidence interval	AIDS adult‡ central tendency estimate	AIDS adult‡ 95% confidence interval	AIDS pediatric central tendency estimate	AIDS pediatric 95% confidence interval
pD, p(diarrheal illness | infection)	0.40	0.20-0.80	0.70	0.35-1.00	0.95	0.80-1.00	0.95	0.80-1.00
pM, p(moderate-severe illness | D)	0.15	0.08-0.30	0.20	0.10-0.40	0.95	0.80-1.00	0.95	0.80-1.00
pV, p(physician visit | M)	0.33	0.17-0.66	0.50	0.25-0.75	0.90	0.70-1.00	0.95	0.80-1.00
pO, p(ova & parasite exam | V)	0.25	0.10-0.63	0.50	0.20-0.75	0.90	0.70-1.00	0.95	0.80-1.00
pC, p(Cryptosporidium test | O)	0.10	0.05-0.20	0.15	0.08-0.30	0.95	0.80-1.00	0.95	0.80-1.00
pR, p(case detection & reporting | C)	0.60	0.40-0.80	0.60	0.40-0.80	0.95	0.80-1.00	0.95	0.80-1.00

* See methods section for definitions of terms and explanations of derivations

† Lognormal distributions were assumed for probabilities in non-AIDS subgroups with k = 2 for all probabilities except p0 (k = 2.5) and pR (k = 1.5)

‡ Probabilities for AIDS subgroups were subjected to arcsine transformations to avoid truncation with lognormal distributions; 95% confidence intervals were calculated on transformed data with standard deviations of 7 degrees (for p = 0.95) and 7.5 degrees (for p = .90) (Zar, 1984)

attributable diarrhea (Wuhib *et al.* 1994). For the AIDS population, both pediatric and adult, a probability of 0.95 (95 percent CI 0.80-1.00) was applied.

p(moderate-severe illness | diarrhea), pM. Morbidity in the moderate-severe range is defined as diarrheal illness (with or without other symptoms) that has resulted in severe impairment (e.g., debilitation and/or dehydration) or moderate-severe impairment which is not improving after about one week. In the volunteer study, of the seven diarrheal subjects, three shed oocysts for seven or more days, but the maximum duration of clinical diarrhea was only four days, and none of the illnesses were characterized as severe (Chappell *et al.* 1996; DuPont *et al.* 1995). A community survey performed in the Milwaukee outbreak showed that the duration of watery diarrhea ranged from 1 to 38 days with a median of 3 days among adults who experienced this symptom (Mac Kenzie *et al.* 1994). An estimate of pM of 0.15 (95 percent CI 0.08-0.30) was made based on this information. For the pediatric population, it was assumed that the likelihood of moderate-severe morbidity will be about one-third higher than in adults, or 0.2 (95 percent CI 0.10-0.30). This is supported by findings which showed a greater susceptibility to more severe illness among neonatal and very young subjects (Current and Garcia 1991; O'Donoghue 1995). For the adult and pediatric AIDS populations, we considered the observations that *Cryptosporidium* infections occurring in persons with CD4 counts below 180 are generally not self-limiting (Flanigan *et al.* 1992). A value of 0.95 (95 percent CI 0.80-1.00) was selected for pM in this population, consistent with observations that most *Cryptosporidium* infections in persons with AIDS are severe and/or chronic.

p(physician visit | moderate-severe illness), pV. Given the presence of moderate-severe gastrointestinal symptoms, we are interested in the probability that the condition will receive medical attention from a physician. Other physician contacts may occur, as less severe cases may seek care and telephone contacts may occur as well, but these other instances are unlikely to result in an O&P examination. Physician contact for diarrhea is likely to be influenced by factors reflecting illness severity, access to care, and other personal factors such as tendencies or biases toward seeking physician care. As an example, it has been shown that among populations meeting clinical definitions of chronic irritable bowel syndrome, less than half reported ever having seen a physician for the condition (Talley *et al.* 1995). An additional consideration which may decrease the tendency to seek and receive in-person care even in those with moderate-severe *Cryptosporidium* infection are the frequent absences of systemic illness, fever, and abdominal pain (Mac Kenzie *et al.* 1994; Public Health Laboratory Service Study Group 1990). For the adult non-AIDS population, pV was estimated to be one-third (0.33, 95 percent CI 0.17-0.66). It was estimated that children are approximately 50 percent more likely than adults to visit a physician as a result of moderate-severe enteric infection; this provides a 0.5 probability (95 percent CI 0.25-0.75).For the adult and pediatric subgroups, respectively, probabilities of 0.9 (95 percent CI 0.80-1.00) and 0.95 (95 percent CI 0.70-1.00) were selected because AIDS patients are generally under a regular and frequent schedule of care and the diarrheal symptoms are likely to be prolonged and therefore present when a visit occurs.

Given that a diarrheal illness has occurred, the combined probabilities of moderate-severe illness (pM), and physician visits (pV) provide the following summary probabilities for visits to a physician in the non-AIDS subgroups: 5 percent for adults and 10 percent for children. These figures are in accord with other available estimates. In Milwaukee, 6.5 percent of adults in the general population who had experienced watery diarrhea reported visiting a physician (Mac Kenzie *et al.* 1994). A study of diarrheal illness in children less than five years old estimated that approximately 9 percent of episodes resulted in physician contact (Glass *et al.* 1991). Lastly, it was estimated that physician contacts were sought in eight percent of acute cases of infectious intestinal disease in the U.S. (Garthright *et al.* 1988).

p(ova & parasite exam | physician visit), pO. The next event in the sequence is the ordering and submission of a stool sample for an O&P exam, which may or may not include testing for *Cryptosporidium*, as described in the next section. The probability of this occurring (pO), given that a patient with moderate-severe diarrheal illness is seen by a physician, might be expected to be somewhat high, as the preceding series of events were constructed in such a way that it might be reasonable for a physician to request O&P testing. Decreasing the likelihood that an O&P will be ordered are the possibilities that: a) a supportive approach might be taken, with subsequent improvement; b) other testing would be done first, with subsequent improvement; c) the absence of unusual circumstances (e.g., foreign travel, camping, etc.) which might arouse clinical suspicion of parasitic infection; and d) questions regarding the cost effectiveness and value of stool testing (Chitkara *et al.* 1996). In addition, even if a physician orders an O&P test,

ambulatory patients may not submit requested samples, or may do so in an inappropriate or untimely manner.

For the non-AIDS adult group, we estimated that one-quarter (0.25, 95 percent CI 0.10-0.63) will have O&P exams performed. A probability of 0.50 (95 percent CI 0.20-0.75) was selected for non-AIDS pediatric group because children may receive a more aggressive diagnostic approach (Laney and Cohen 1993) and suspicion of parasitic infection may be aroused coincidentally in young patients attending day-care, as this is a widely-recognized setting for parasite transmission (Cordell and Addiss 1994; Laney and Cohen 1993). In persons with HIV infection, aggressive testing for an etiologic agent of chronic diarrhea has been described and recommended (Dieterich *et al.* 1994; Park and Giannella 1993; Smith 1993); probabilities of 0.9 (95 percent CI 0.70-1.00) and 0.95 (95 percent CI 0.80-1.00) were selected for the adult and pediatric AIDS subgroups, respectively.

p(Cryptosporidium test | ova & parasite exam), pC. Testing for *Cryptosporidium* as part of the O&P exam may be expected to depend primarily upon physician and patient characteristics, diagnostic laboratory policies, and state/local requirements. The primary benefit of diagnosis from the clinical perspective may be the exclusion of other causes, as no drug therapies for *Cryptosporidium* have proven effective (Blagburn and Soave 1997). Awareness among physicians of the symptoms of cryptosporidiosis is not universal, and many physicians may mistakenly assume that it is a routine component of the O&P (Morin *et al.* 1997). Evidence strongly suggests that it is likely that in the majority of

O&P exams for patients who are not HIV-positive, *Cryptosporidium* testing will not be requested.

In the U.S., only five percent of laboratories performing O&P exams routinely screen for *Cryptosporidium* (Boyce *et al.* 1996). An additional seven percent of surveyed laboratories cited liquid stool specimens as an indication for them to perform *Cryptosporidium* screening (Boyce *et al.* 1996). There have also been general recommendations that children be targeted for *Cryptosporidium* screening (Current and Garcia 1991; Maguire *et al.* 1995). Probabilities of 0.1 (95 percent CI 0.05-0.20) and 0.15 (95 percent CI 0.08-0.30) were selected for the non-AIDS adult and pediatric cases, respectively. In persons with AIDS, a probability of 0.95 (95 percent CI 0.80-1.00) was selected for *Cryptosporidium* testing, because most physicians treating AIDS patients are aware of cryptosporidiosis as an opportunistic disease affecting the medical management of their patients.

p(case detection and reporting| Cryptosporidium test), pR. The likelihood that a cryptosporidiosis case will be diagnosed and reported to the appropriate health authority subsequent to a stool exam for *Cryptosporidium* depends on the sensitivity of the testing and the effectiveness of the reporting system. The overall sensitivity of testing will be affected by the number of exams performed, the intermittence, duration, and intensity of oocyst shedding, and the laboratory's diagnostic sensitivity and proficiency. The volunteer study demonstrated that for the seven infected subjects with diarrheal illness, the median percentage of positive stools during illness was 62.5 percent (Chappell *et al.*

1996). Compared to this research setting, most clinical laboratories use a less sensitive detection technique (Boyce *et al.* 1996), and may also be more time-constrained or less proficient. This could be offset by instances in which multiple samples are submitted (as per the general recommendation that up to three tests be performed if parasites are strongly suspected). The presence of an active surveillance system that is essentially 100 percent effective at soliciting case reports was assumed. Therefore, a value of 0.6 (95 percent CI 0.40-0.80) was assigned to pR for the non-AIDS populations. For persons with AIDS, there is an increased likelihood that repeat samples will be submitted and that oocyst shedding will be heavy and persistent; a study showed that that nearly 100 percent of AIDS-related infections were detected with one or two samples (Clavel 1995). For both AIDS groups, we ascribe probabilities of 0.95 (95 percent CI 0.80-1.00) to pR.

d. Observed disease levels

At this time, the only data on the incidence of endemic *C. parvum* infection in the U.S. come from states where cryptosporidiosis is a reportable disease or from AIDS surveillance programs. Surveillance generally relies on reporting from laboratories that perform stool examinations and/or from health care providers (Frost *et al.* 1996). New York City was one of the first public health authorities in the U.S. to mandate reporting of cryptosporidiosis (New York City Department of Environmental Protection 1996). Under the auspices of the New York City Department of Health, active laboratory-based surveillance for cryptosporidiosis has occurred since November 1994. Basic demographic data are ascertained from laboratory and physician records, and are supplemented by case-patient interviews. While no outbreaks have been recognized,

approximately 35 to 40 new cases were identified monthly during the first two years of the program (New York City Department of Environmental Protection 1996), mostly in persons with HIV infection (Miller *et al.* 1997). Among persons with AIDS, rates of cryptosporidiosis in New York City have generally been similar to national rates (New York City Department of Health 1996).

Chronic cryptosporidiosis of greater than one month's duration is an AIDS-defining condition (Centers for Disease Control and Prevention 1992). The current surveillance definition of AIDS includes persons who have HIV infection and CD4 counts (cells/μl) lower than 200 or the diagnosis of a specific opportunistic infection (Centers for Disease Control and Prevention 1992). Therefore, it is likely that most HIV-positive persons diagnosed with cryptosporidiosis meet the surveillance definition for an AIDS case. A partial summary of data for 1995 is presented in Table 2-3.

3. RESULTS

Table 2-4 summarizes the output from the exposure-infection component of the model. Estimates of the annual risk of infection to individuals from exposure to *C. parvum* via tap water are presented for each of the four subgroups in Table 2-4, part A. In the non-AIDS subgroup, the median annual risk of infection is close to one in a thousand at the unit concentration; risks associated with other concentrations can be calculated by multiplying by the ratio of the concentration of interest and the unit

Table 2-3: Approximated cryptosporidiosis surveillance results, by AIDS status and age group, New York City, 1995

age group	non-AIDS related cases (%)	AIDS related cases (%)	combined
adult	40 (9)	390 (83)	430 (91)
pediatric	30 (6)	10 (2)	40 (9)
all ages	70 (15)	400 (85)	470 (100)

* These data were estimated from:
- the reported proportions of cases occurring in persons with HIV infection in the <20 and ≥20 years age classes (25% and ≥91%, respectively; J. Miller *et al* ., APHA, 1996), with the assumption that most cases with HIV infection meet the AIDS case definition, since cryptosporidiosis is an AIDS defining condition (CDC, 1992), and
- the reported proportions of cases occurring in the 0-9 years and 10-19 years age groups, linearly apportioned to 10-12 years (NYCDEP, 1996)

Table 2-4: Predicted annual risks and numbers of *Cryptosporidium* infection from consumption of tapwater with unit concentration (0.001 oocyst/L) for non-AIDS and AIDS subgroups

A. Predicted infection risk (infection/person/year):

subgroup	central tendency	95% confidence interval	dispersion factor, k
non-AIDS Adult	0.0009	0.0003 - 0.0028	3.2
non-AIDS Pediatric	0.0008	0.0002 - 0.0024	3.2
AIDS Adult	0.0019	0.0003 - 0.0130	6.9
AIDS Pediatric	0.0016	0.0002 - 0.0110	6.9

B. Predicted annual infections in the sample population:

subgroup	central tendency	95% confidence interval	dispersion factor, k
non-AIDS Adult	5,400	1,700 - 17,000	3.2
non-AIDS Pediatric	940	300 - 3,000	3.2
AIDS Adult	56	8 - 400	7.0
AIDS Pediatric	1	0 - 9	7.2

concentration. Infection risks for the AIDS subgroups are approximately double those in the respective non-AIDS subgroups, reflecting the assumptions regarding relative tap water avoidance and increased susceptibility to infection. Wider confidence intervals in the estimates for AIDS subgroup follow from the increased uncertainties associated with these assumptions. At the lower end of the confidence intervals, risks are similar across the subgroups. At the upper end, the AIDS estimates are equivalent to about a one percent annual risk, a level which approaches available estimates of the incidence of cryptosporidiosis in this group (Juranek 1995; Pedersen *et al.* 1996). The fact that low risks affecting large populations can result in moderate impacts is also illustrated (Table 2-4, part B); more than 6,000 infections are estimated if the risks associated with the unit concentration are applied to the New York City population, with approximately 99 percent occurring in the non-AIDS categories.

The estimates of the overall probabilities that an infection will result in a reported case demonstrate the expected divergence between the non-AIDS and AIDS subgroups (Table 2-5). Only about three reported illnesses out of 10,000 infections occurring in non-AIDS adults are predicted (95 percent CI 5.4×10^{-5}-1.6×10^{-3}). In comparison, for the pediatric non-AIDS subgroup, the overall effect of the moderately higher estimates of the component probabilities was an approximately 10-fold higher estimate of the probability of a case report. Uncertainties in these estimates for the non-AIDS subgroups are substantial, as reflected in the two orders-of-magnitude ranges in the confidence intervals. However, the estimates appear reasonable when compared to information from outbreak situations. In contrast to the results for the non-AIDS subgroups, the analysis

predicts that the majority of infections occurring in persons with AIDS are likely to result in case reports, with the confidence intervals encompassing a plausible range of estimates (e.g., adults, 95 percent CI 0.39-0.80).

The overall results combining the exposure-infection and infection-outcome components are presented in Table 2-6, which summarizes the numbers and proportions of tap water-related cases per year by age class and AIDS status estimated to occur in the sample (New York City) population, following exposure at the unit concentration.

In comparing the model output (Table 2-6) to surveillance data for cryptosporidiosis in the sample population (Table 2-3), we must recognize that we do not know what the actual occurrence of infective *C. parvum* in tap water was nor the fraction of endemic cases which are actually due to tap water consumption. In terms of magnitude, the central tendency estimates corresponding to the unit concentration for the overall population and its components are approximately five to ten percent of the surveillance figures; the confidence interval in the number of total predicted cases represents about two to fifty percent of the total cases detected by cryptosporidiosis surveillance in 1995. A comparison of the predicted proportions of cases by age and AIDS status (Table 2-6B), shows that the model estimates are quite similar to the surveillance data. These patterns are dominated by the high proportion of cases occurring in adults with AIDS, which was close to 85 percent for both the model and the surveillance data. This prediction depends on the actual population of persons in this category, and in the model this figure is most sensitive to the assumption regarding the relative susceptibility of persons with AIDS to infection.

Table 2-6: Overall model results showing numbers and proportions of cases by age class and AIDS status calculated at the unit concentration (0.001 oocysts/liter)

A. Number of calculated tapwater-related cases per year*

	non-AIDS			AIDS			combined	
age group	central tendency	95% confidence interval	dispersion factor, k	central tendency	95% confidence interval	dispersion factor, k	central tendency	95% confidence interval
adult	2	0-13	7.9	33	5-240	7.3	36	6-240
pediatric	3	0-23	7.9	1	0-6	7.5	4	1-25
all ages	6	1-29		34	6-240		42	10-250

B. Proportions of tapwater-related cases per year by age class and AIDS status†

	non-AIDS		AIDS		combined	
age group	proportion (%)	95% confidence interval	proportion (%)	95% confidence interval	proportion (%)	95% confidence interval
adult	4	0-35	85	29-98	90	40-99
pediatric	8	0-53	2	0-20	10	1-60
all ages	12	1-66	88	34-99		

* Numbers of cases for all categories are from the distribution of results of Monte Carlo simulations ($n \geq 6{,}000$); numbers for all ages and/or combined AIDS status are not equivalent to marginal totals.
† Proportions are based on average numbers of cases occurring in each category with confidence intervals based on the distribution of results.

Reviewing the uncertainties in the model, as indicated by the dispersion factors (k) in tables 2-4 through 2-6, it is apparent that much of the uncertainty in the AIDS subgroups is contributed by the exposure-infection component (Table 2-4), while for the non-AIDS subgroups more uncertainty is contributed by the infection-outcome component (Table 2-5). However, the overall uncertainties (Table 2-6) are similar for the different subgroups, with approximate 50 to 60-fold ranges in the confidence intervals (obtained from k^2) for all four subgroups. At present, these uncertainties are overshadowed by the large degree of uncertainty associated with the concentration parameter, which was described earlier as taking at least a 100-fold range over its 95 percent confidence interval.

4. DISCUSSION

In this study, we used a risk assessment model to examine the potential role of tap water in the transmission of endemic *C. parvum* infection. The present study differed from previous approaches in two fundamental ways: a) the exposed population was divided according to AIDS status and age, and b) the probabilities of outcomes leading from infection all the way to case reporting were quantified. The model output was generally reconcilable with available data. Previous risk assessments of *Cryptosporidium* in drinking water reported mixed results when attempting to compare predicted risks with observed disease rates, reflecting, in part, difficulties associated with interpreting infection as an endpoint (Haas and Rose 1994; Kissel *et al.* 1996). The approach taken in this paper, particularly the characterization of host susceptibility and the consideration of the spectrum of clinical manifestations, represents changes in the traditional chemical risk

assessment paradigm which are desirable in the assessment of risks from pathogens (ILSI Risk Science Institute Pathogen Risk Assessment Working Group 1996).

The analysis was consistent with and lends support to the premise that low level transmission via tap water can represent an important exposure route for endemic *Cryptosporidium* infection, even at very low levels of occurrence of the pathogen in drinking water. Secondary transmission was not incorporated in our model, as it has not been shown to play a large role in outbreaks, but it could increase the importance of low level endemic transmission. There is a clear need for improved epidemiologic investigations of the role of tap water relative to other exposures, especially for persons with AIDS (Centers for Disease Control and Prevention 1995).

Although the uncertainties inherent in our risk assessment are substantial, it was shown that the uncertainty contributed by the concentration parameter remains dominant; this finding was consistent with other risk assessments for waterborne pathogens (Eisenberg *et al.* 1996; Kissel *et al.* 1996; Teunis *et al.* 1997). The model was evaluated at a unit concentration, the magnitude of which is consistent with, but not equivalent to, data derived from *Cryptosporidium* monitoring performed to date in the U.S. The application of new monitoring technologies is likely to provide improved characterizations of the relevant concentration, reducing the uncertainty inherent in a risk assessment of this type.

The analysis presented here offers further demonstration of the manner in which the disciplines of risk assessment and epidemiology may inform one another (Eisenberg *et al.*

1996). The model presented in this paper made extensive use of descriptive epidemiologic data. Risk assessment cannot by itself demonstrate cause and effect, but may provide support to epidemiologic efforts in evaluating plausibility and helping to formulate relevant questions, as well as assisting in the design of epidemiologic investigations. For example, the model demonstrated how surveillance for detected cases of a reportable illness may substantially underestimate rates of infection and morbidity. As has been noted, investigations of the incidence of *Cryptosporidium* infection (or other enteric infections with a wide spectrum of clinical manifestations) may usefully focus on serological evidence of exposure and infection (Centers for Disease Control and Prevention 1995).

Since there are no treatments for cryptosporidiosis, reduction of exposure is crucial for those persons most susceptible to severe outcomes following infection. The analysis presented here supports recommendations for clear general advisories for the immunocompromised regarding the avoidance of unboiled tap water (Meinhardt *et al.* 1996).

The relative susceptibility of persons with AIDS to infection (or to chronic outcomes following infection) may change over time, and we may be witnessing this currently. The number of reported cases of cryptosporidiosis in New York City and throughout the U.S. generally has dropped since the end of 1996 (Centers for Disease Control and Prevention 1996b; Centers for Disease Control and Prevention 1997a; New York City Department of Environmental Protection 1997). This reduction coincides with

significant declines in the death rate from AIDS (Centers for Disease Control and Prevention 1997b), which has been attributed to improved combination drug therapies against HIV (Detels *et al.* 1998). It is possible that there has been a decline in the number of *Cryptosporidium* infections in the AIDS population and/or that outcomes are less severe (e.g., asymptomatic, mild or self-limiting) in hosts whose immune status is no longer characterized by an inexorable decline (Foudraine *et al.* 1998; Carr et al. 1998). These welcome developments may indicate that some of the assumptions used in our model would require revision for applicability to the current situation, such as the use of the AIDS population in a community as a surrogate for the number of persons at risk for severe illness resulting from *Cryptosporidium* infection.

Perhaps the most interesting finding of this analysis was the indication that the observed patterns of disease could result from an exposure common to the entire population. This holds true irrespective of whether tap water is a major source. In other words, the higher levels of cryptosporidiosis that have been observed in persons with AIDS do not necessarily require a unique or unusual exposure route. Generalizing, the record of a disproportionate occurrence of a condition in a particular subgroup of the population does not automatically implicate a specific or exclusive exposure.

Note: the study described above was published as:

> Perz JF, Ennever FK, Le Blancq SM. *Cryptosporidium* in tap water: comparison of predicted risks to observed levels of disease. American Journal of Epidemiology 147:289-301(1998).

III. *CRYPTOSPORIDIUM* GENOTYPING: WILDLIFE SURVEY

A. Introduction

The risk assessment models developed in the previous chapter supported the premise that low level transmission via tap water can represent an important exposure route for endemic *Cryptosporidium* infection, even at very low levels of the pathogen in drinking water (Perz *et al.* 1998). Surface water supplies are vulnerable to *Cryptosporidium* contamination from a variety of sources. Watershed protection programs attempt to control such inputs from anthropogenic and agricultural sources (Ashendorff *et al.* 1997), but wildlife remain largely beyond management efforts. Numerous mammalian species of wildlife have been shown to be infected with *Cryptosporidium* spp (O'Donoghue 1995). *Cryptosporidium* infection has been reported in both captive and wild white-tailed deer populations in the mid-Atlantic and southern U.S. (Fayer *et al.* 1996; Rickard *et al.* 1999). White-tailed deer are of particular concern in the eastern U.S. because of their high population densities in both rural and suburban areas, including the watersheds of New York City's Catskill-Delaware and Croton water supply systems.

It was determined that a survey of wildlife in lower New York State, focusing primarily
ıite-tailed deer, should be performed to assess whether they may be an
ınmental source of *Cryptosporidium*. Characterization of *Cryptosporidium*-positive
s with respect to species and strain was deemed critical in order to evaluate
:r wildlife are sources of *Cryptosporidium* relevant to humans. The goal of

performing molecular characterization on *Cryptosporidium* isolates itself involved two prerequisites: (1) characterization of ribosomal DNA (rDNA) to allow speciation and typing and (2) the development of PCR-based methods and associated reagents. These goals were formulated as the following specific aims:

Specific Aim #2A – To characterize the *C. parvum* rDNA loci from a typical human genotype isolate for comparison with a typical bovine genotype isolate and the evaluation of intra- and interspecies differences.

Specific Aim #2B – To develop an assay for the detection of *Cryptosporidium* in feces which improves upon and expands current diagnostic capabilities by using strategies based on the polymerase chain reaction (PCR), tailored for samples from asymptomatic wildlife.

Specific Aim #2C – To investigate the potential role of wildlife as environmental sources of *Cryptosporidium* within drinking water basins by performing a survey of wildlife for the presence of *Cryptosporidium* in their feces, focusing on white-tailed deer.

Specific Aim #2D – To characterize isolates of *Cryptosporidium* identified in wildlife and compare these with isolates from cases of human cryptosporidiosis.

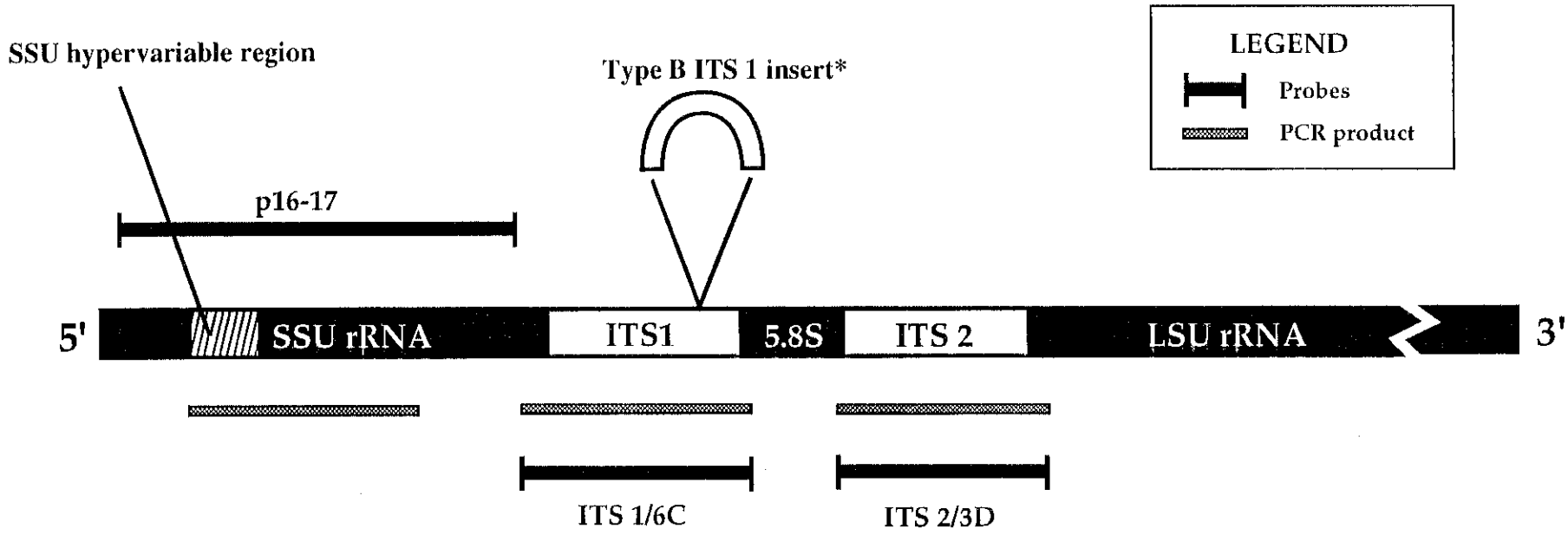
The remainder of this chapter is organized into two sections which follow below. Section B relates to Specific Aim #2A while section C relates to Specific Aims #2B-D.

B. Characterization of the rDNA loci

1. RATIONALE

Accurate interpretation of data from PCR-based characterization studies involving rDNA from different *Cryptosporidium* isolates rests on the proper characterization of these genes. Ribosomal RNA genes have been used extensively to identify isolates and examine the phylogenetic relationships among species (Sogin *et al.* 1989). The genes encoding the cytoplasmic ribosomal RNAs in most higher organisms (i.e. eukaryotes) are organized into transcriptional units containing a small subunit (SSU) rRNA gene, a 5.8S rRNA gene, and a large subunit (LSU) rRNA gene in a 5'-3' orientation separated by internal transcribed spacers. These rDNA units are often present as hundreds or even thousands of copies with virtually identical sequences; this homogeneity is maintained by frequent recombination events, made possible by the organization of these genes into large tandem arrays (Klein and Petes 1989; McCutchan *et al.* 1995). Their homogeneity allows for the comparison of sequences between organisms with little concern for the complications of intra-organismal heterogeneity. Certain protozoa violate this paradigm, as their rDNA units are few in number and are dispersed throughout the genome

(McCutchan *et al.* 1995). It was demonstrated for the bovine genotype that *C. parvum* falls into this alternate category (Le Blancq *et al.* 1997). Furthermore, of the five copies of the rDNA units, four were of one type (Type A) while the other was of a different type (Type B), with the major difference apparent in the size and sequences of the internal transcribed spacers (ITS) (Le Blancq *et al.* 1997). Numerous other sequence differences were detected in other regions of the rDNA units as well (Le Blancq *et al.* 1997). The presence of such heterogeneities may lead to the misinterpretation of inter-organism sequence differences (Carraway *et al.* 1996). It is therefore of interest to determine whether the rDNA loci in the human genotype of *C. parvum* are similarly organized into two types. A strategy was developed to clone and sequence the internal transcribed spacer regions and the hypervariable region of the SSU rRNA gene of the rDNA units from the human *C. parvum* genotype (Figure 3-1). This would demonstrate whether there are two distinct rDNA units in the human genotype as well as determining the sequence heterogeneity between the two genotypes. An understanding of the comparative organization of these genes will facilitate the characterization and comparison of strains and their phylogenetic relationship at, above, and below the species level.



Experimental Design:

(a) Identify phage clones containing rDNA sequences through screening with the SSU rDNA gene probe.
(b) Amplify regions of interest - ITS1, ITS2, and hypervariable region - through the PCR.
(c) Clone and sequence the various PCR products.

* the Type B ITS1 is approximately 500bp longer than the Type A ITS1.

Figure 3-1: rDNA Units- Schematic Representation of Experimental Plan

2. MATERIALS AND METHODS

a. Genomic Libraries and Probes

The SFGH1 Lambda Gem11 library, a *C. parvum* library prepared from partially-digested genomic DNA extracted from a human genotype isolate (SFGH1) (provided by Dr. R. Nelson, UCSF), was screened by plaque hybridization. Standard protocols were used throughout for the manipulation of phage vectors (Ausubel *et al.* 1995). The library was transfected into the bacterial host KW251, plated, and lifted onto nylon filters. The DNA probe used for screening was the Small Subunit rRNA (SSU rRNA) clone, p17-16, a 1689 bp PCR product amplified from the *C. parvum* isolate GCH1 (bovine genotype) (Tzipori *et al.* 1994), and cloned into pCRII (provided by Dr. G. Widmer, Tufts University). The SSU rRNA clone starts at position 31 and terminates at position 1720 of the complete SSU rDNA sequence deposited by Pieniazek *et al* in the GenBank™ data base under accession number L16996. The DNA probe was prepared from a gel-purified plasmid fragment using random priming with [α^{32}P]dATP (NEN Research Products).

b. Phage Plaque Screening

Standard methods were used for colony hybridization and Southern analysis (Ausubel *et al.* 1995). Overnight hybridization was performed at 65°C and the stringency of the final post-hybridizational wash was 0.1x SSC at 65°C. The hybridization signal was monitored by autoradiography. Positive plaques were transferred from the plates to SM buffer.

c. Polymerase Chain Reaction (PCR)

PCR-amplification of the internal transcribed spacers. Two sets of oligonucleotide primers were designed to amplify the ITS regions of the rDNA unit, based on the L16996 sequence described above and the Type A rDNA unit sequence deposited by Le Blancq *et al* in GenBank™ under accession number AF015773. The first set of primers amplified a region spanning ITS1 from the 3' end of the SSU rRNA gene to the 5' end of the 5.8S rRNA gene (See Figure 3-1), with an expected product length of 723 bp from a Type A rDNA unit. The sequence of the forward primer (ITS1F) was 5'-ACACCGCCCGTCGCTCCTA-3' and the sequence of the reverse primer (ITS1R) was 5'-CACTTTGCTGCGTCCTTCATCGTT-3'. The second set of primers amplified a region spanning ITS2 from the 3' end of the 5.8S rRNA gene to the 5' end of the LSU rRNA gene, with an expected product length of 662 bp. The sequence of the forward primer (ITS1F) was 5'-ATCTCTCAACGCAAATAGCAGTAAT-3' and the sequence of the reverse primer (ITS1R) was 5'-TTTTCCTGTTCACTCGCAGTTA-3'. PCR-amplification was performed directly on the phage stocks (Cheng *et al.* 1994) as per the protocol shown in Table 3-1. Following an automated hot start, 30 cycles were performed with a combined annealing-extension step at 68°C. Visualization and sizing of PCR products was performed by electrophoresis in agarose gels pre-stained with ethidium bromide. No PCR products were seen in control reactions without template.

A representative PCR product characterized by a single clear band of the expected size was selected for both ITS1 and ITS2 (plaques #6C and #3D respectively, data not

Table 3-1: Protocol for PCR-amplification of DNA from Phage Stocks
(Modified from Cheng *et al*, 1994)

A. In area designated for PCR set-up, assemble the following reagents and associated materials including dedicated pipettors and filtered pipette tips.

Reagent	Volume (µl)	Final Concentration (in 50 µl Reaction)
Sterile H_20	23.75	---
10x PCR Buffer	5	1x
DNTP mix (d-A,C,G,T-TP)	4	0.2 mM each
$MgCl_2$, 25 mM	6	3.0 mM
Forward Primer	5	0.5 mM
Reverse Primer	5	0.5 mM
Taq DNA polymerase	0.25	1.25 units per reaction
Template (e.g. λ phage in SM)	1	~10^7 phage per reaction
Total	50	

B. Note that each experiment must include at least one negative control without template DNA and should also include a positive control consisting of genomic DNA or a reliable λ phage plaque.

C. Prepare fresh Master Mix of all the above reagents except template, adding reagents in the order listed. Scale volumes appropriately for the number of reactions planned. Mix tube by inversion and then centrifuge briefly.

D. Label 0.5 ml reaction tubes and arrange in rack(s).

E. Add approximately 60 µl mineral oil to each tube.

F. Add 49 µl master mix to each tube.

G. Relocate to designated area for handling phage/clones.

H. Mix thoroughly by gently tapping tubes containing λ phage plaques in SM buffer and add 1 µl template to each reaction tube, taking care to avoid cross-contamination.

I. Centrifuge the reaction tubes briefly at low speed (e.g. 30 sec @ 1000 g) to collect the contents at the bottom of the tube.

J. Place the reaction tubes in the center of the PCR machine after adding one drop of mineral oil to the appropriate wells.

(continued on next page)

Table 3-1: Protocol for PCR-amplification of DNA from Phage Stocks (continued)

K. Perform thermal cycling as per the following conditions:

1) 9 min @ 95°C (*Taq* activation)
2) 30 cycles of:
   (a) 45 sec @ 94°C (denaturing)
   (b) 150 sec @ 68°C (annealing-extension)*
3) 7 min @ 72°C (final extension)
4) 4°C hold

L. Examine the PCR reaction products by gel electrophoresis. Load an aliquot of each PCR reaction (10-15 µl) on a 1.0% agarose gel along with appropriate size markers (e.g. 1 kb ladder). Examine and photograph under UV light.

M. Blot gel on to nylon filters for Southern analysis as required.

N. Store PCR products at –20°C.

* Note that for amplifications with the SSUV primer set, annealing and extension occurred as separate 45 sec steps at 60°C and 72°C respectively

Materials:

Thermal Cycler 480 (PE Applied Biosystems)
0.5 ml thin-walled reaction tubes (PE Applied Biosystems)
AmpliTaq Gold DNA polymerase (PE Applied Biosystems)
mineral oil (Sigma)

shown). These were cloned into the PCRII plasmid vector using the TA cloning kit (Invitrogen).

Southern Analysis of PCR products. Standard methods were used for the Southern analysis of the ITS1 and ITS2 PCR products, which had been transferred from the gels onto nylon filters. The cloned PCR products, ITS1/6C9 and ITS2/3D18, were prepared as DNA probes by gel-purification of the plasmid inserts and random priming with [α^{32}P]dATP (NEN Research Products). Overnight hybridizations were performed at 50°C. A series of post-hybridization washes was performed at increasing stringencies, ranging from 3xSSC @ 50°C to 0.1xSSC @ 65°C, with monitoring of the hybridization signal by autoradiography after each wash.

d. Detection of Putative Type B ITS Sequence

One of the plaques, # 3C, showed evidence of a larger than expected band for a Type A rDNA unit on the autoradiographs of the ITS1 PCR products. This band, which was not visible on the ethidium bromide-stained gel, appeared to be in the ~1200 bp range, consistent with the size of the Type B ITS1 previously characterized for the bovine genotype. PCR amplification of plaque 3C was repeated using both primer sets, 5 additional cycles (35 total), and varying levels of template. Positive and negative controls were also included. One of the resulting ITS2 PCR products and one of the ITS1 reactions (which resulted in a ~1200 bp product) were cloned into the PCRII-TOPO plasmid vector using the TOPO cloning kit (Invitrogen). The resulting clones were designated as ITS1/3C16 and ITS2/3CP1.

e. PCR-amplification of the Hypervariable Region of the SSU Gene

Two oligonucleotide primers were designed to amplify a hypervariable region of the SSU rRNA gene, with an expected 401 bp product based on the L16996 sequence. The sequence of the forward primer (SSUV1) was 5'-TCAATTGGAGGGCAAGTCTGGTG-3' and the sequence of the reverse primer (SSUV2) was 5'-AAACATCCTTGGCAAATGCTTTCG-3'. PCR conditions were as described in Table 3-1. Templates consisted of plaques 3C, 3D, and 6C along with positive and negative controls. A representative product for each of the three plaques was then cloned into the PCRII-TOPO plasmid vector.

f. Sequence Analysis

Sequencing was performed on both DNA strands for each clone using forward and reverse M13 primers. In addition, for the ITS clones, sequencing was also performed with the PCR primers. The nucleotide sequences of these clones were determined by the dideoxy-chain termination method through automated sequencing. Sequence analysis and alignments were carried out with DNA Strider and GCG software (Madison, WI). Searches of the DNA databases were performed with the BLAST (Basic Local Alignment Search Tool) program (Altschul *et al.* 1997).

3. RESULTS

Screening of the SFGHI Lambda Gem11 Library resulted in the identification of 53 positive plaques of varying signal intensities. Phage plugs corresponding to all of these positive plaques were used as templates for PCR-amplification with the ITSI and ITS2 primers. Of the 53 phage plaques, 20 resulted in visible ITS1 products and 16 resulted in visible ITS2 products. When visualized with ethidium bromide staining, all of the resulting PCR products appeared to be of the expected size based on the bovine genotype Type A sequence. Therefore, Southern analysis was undertaken with the aim of detecting anomalous products that might suggest the presence of more than one type of rDNA unit. Such products would be evidenced by product size heterogeneities detectable with the increased sensitivity afforded by Southern analysis or by hybridization signals not correlated with the intensity of the ethidium bromide visualized bands, perhaps reflecting significant sequence differences. This strategy was successful in identifying a phage plaque (#3C), which had resulted in a larger than expected ITS1 product, as described above.

Analysis of the four ITS clones confirmed the presence of two distinct types of rDNA units. Clones ITS1/6C and ITS2/3D18 were designated as Type A and clones ITS1/3C16 and ITS2/3Cp1 were designated as Type B. While the two rDNA types are marked by substantial sequence heterogeneities, the defining characteristic which distinguishes them is the presence of a ~500 bp insert at the 3' end of the Type B ITS1. The sequence of this insert was not determined because of apparent interference with the sequencing reaction

by the presence of secondary structure in the ITS1 Type B template. The size, structural anomaly and location of the insert are similar to that seen in the bovine Type B ITS1, where sequencing was likewise interrupted.

Further evidence of secondary structure came from the observation that PCR reactions with the Type B phage (#3C) produced a single clear band of the size corresponding to Type A (720 bp) under certain conditions (higher template concentrations). This suggests the preferential amplification of a smaller PCR product when the secondary structure is not effectively denatured. This observation suggests that additional Type B units may have been present among the 53 phage clones examined. (This study did not address the copy numbers of the Type A and B units, but if one makes the assumption that the bovine genotype paradigm applies, the two types would be expected to occur in the genome at a 4:1 ratio).

Analysis of the ITS1 sequences is complicated by the 500 bp insert in the Type B sequences, for which actual sequence information is absent. However, by performing pairwise comparisons and multiple alignments using the available sequences, it was shown that the sequences were more similar across genotypes than within (Figure 3-2). The greatest similarity was between the two examples of the Type B ITS1.

Alignments of the Type A and Type B ITS2 sequences from the human and bovine genotypes showed numerous differences as illustrated in Figure 3-3. In the flanking 3' 5.8S and 5' LSU regions, only one difference was detected: a tri-nucleotide

Figure 3-2: Alignments of Type A and Type B ITS1 sequences from the human and bovine genotypes. The flanking 5' SSU and 3' 5.8S sequences are indicated by the wavy lines. For both of the Type B units, sequencing was interrupted after nt 349; in addition to the gap shown following that position, the Type B units both also contain a ~500 nt insert (see text for details).

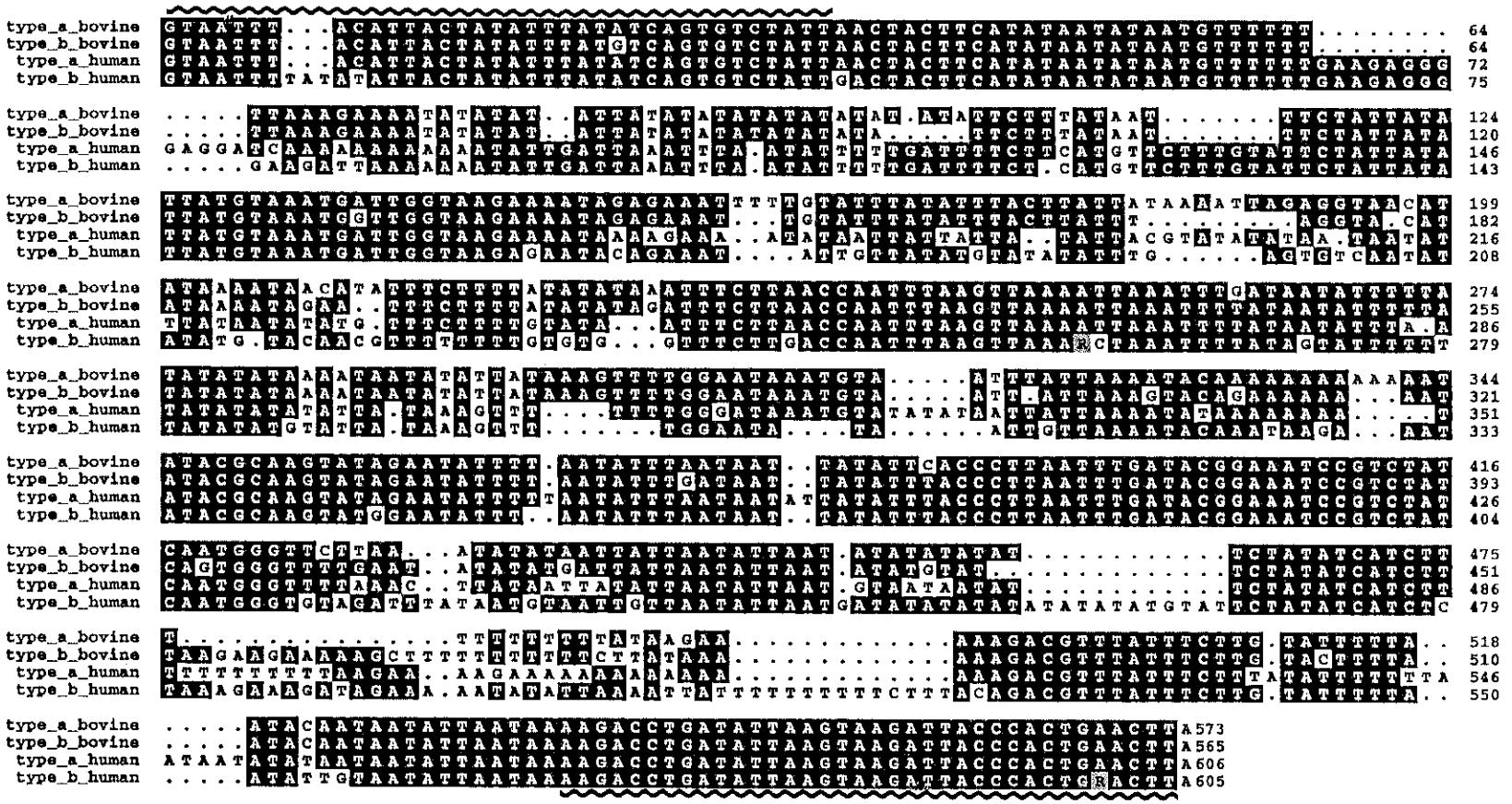


Figure 3-3: Alignments of Type A and Type B ITS2 sequences from the human and bovine genotypes. The flanking 5' 5.8S and 3' LSU sequences are indicated by the wavy lines.

insertion in the 5.8S gene of the Type B human genotype ITS2 sequence. Within ITS2 , the greatest similarity was observed between Types A and B within the bovine genotype.

To further examine the extent to which sequence heterogeneities among the genotypes and rDNA Types extended beyond the ITS regions, analysis of the hypervariable region of the SSU gene (SSUV) was performed. This region represents one of a number of SSU RNA gene regions that display extensive sequence and length variation in all eukaryotes (Gunderson *et al.* 1987). Of particular interest to the present work, other investigators have determined sequences in this region from various *Cryptosporidium* isolates; also, this region is spanned by the SSU primers employed in the screening and characterization phases of this project (described below).

The sequences of the SSUV PCR products from two Type A (phage 3D and 6C) and one Type B (phage 3C) were determined. The two versions of the Type A sequence were identical in this region. The human Type A and Type B were compared with each other and to corresponding sequences available for the bovine genotype (from isolate KSU-1). Alignments of these sequences for both genotypes and both rDNA types reveal a moderate degree of heterogeneity (see Figure 3-4). Differences, in the form of nucleotide substitutions, deletions and insertions, were identified among the four examples. Within each genotype, the Type B unit may be distinguished from its corresponding Type A by a tri-nucleotide deletion (TTT at position 693 and TGA at position 645 for the human and bovine genotypes respectively, with numbering based on

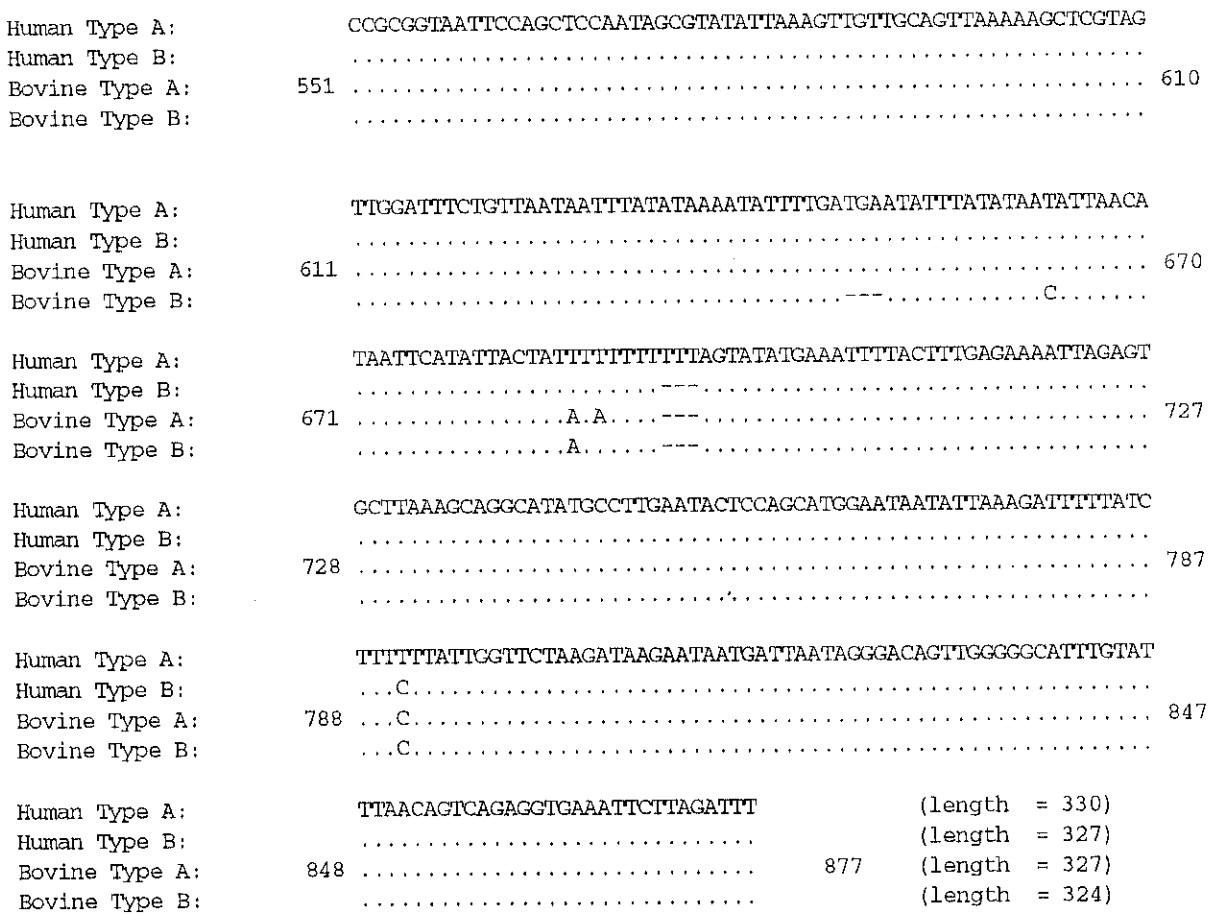


Figure 3-4: Multiple Sequence Alignment of Type A and B rDNA Unit SSUV PCR Products From the Human Genotype (SFGH1) And Corresponding Regions From the Bovine Genotype (KSU-1) Which Include the Hypervariable Region (nt 633-696)

L16996). Additional single nucleotide inter- and intragenotypic differences were present within the region examined in one and two instances for the human and bovine genotypes respectively.

4. DISCUSSION

The finding that two separate types of rDNA unit are maintained in the human genotype, as was previously shown for the bovine genotype, has at least two important implications. First, and of direct relevance to the work described in section C of this chapter, this finding demonstrates the importance of characterizing any targets within the rDNA unit prior to typing isolates or making inferences regarding phylogenenetic relationships. In other words, there is a danger of over-interpreting sequences from the *Cryptosporidium* rDNA unit in the absence of knowledge of Type A and B heterogeneities.

The second implication is that the Type B ITS1 may well have functional significance. One reason for inferring this is that both genotypes have maintained the Type B unit with its additional sequence. In addition, the degree of similarity between the genotypes in the ITS1 Type B regions flanking the additional sequence suggests that mutations in this area have not been tolerated to the extent seen elsewhere in *C. parvum*'s ITS2 or Type A ITS1 (Figures 3-2 and 3-3). The evidence for secondary structure is also in keeping with the hypothesis of functional significance in the Type B ITS1. While this is beyond the scope of the present work, it ought to be addressed directly by further attempts to sequence through this region. This could provide a satisfactory explanation for the maintenance of

the Type B unit and shed some light on its biological significance. A parallel approach that might prove useful would be an assessment of whether the Type B unit is differentially expressed at some point in the parasite's life cycle, following the model of the stage specific expression of different rDNA units in *Plasmodium* (McCutchan *et al.* 1995).

C. Wildlife Survey and Genotyping

A schematic diagram relating to the work performed in this section is presented in Figure 3-5. Samples were collected between January 1996 and July 1998 in two phases. Phase I samples were collected prior to the development of the PCR-based assay and were screened for *Cryptosporidium* using an ELISA method (as described below). The presence of *Cryptosporidium* in all Phase II samples was assayed using the diagnostic PCR assay, the development of which is described in the Methods Development section at the beginning of Materials and Methods. Phase I samples that were ELISA-positive plus selected ELISA-negatives were also assayed using the diagnostic PCR. Subsequently, PCR-positive and selected PCR-negative samples were subjected to PCR-amplification with primer sets that allow typing analysis. Finally, sequence analysis of PCR products was performed.

1. RATIONALE

a. Survey

The rationale for performing this survey for *Cryptosporidium* infection in wildlife was discussed earlier. Briefly, wildlife may represent a reservoir of *Cryptosporidium* infection that pose risks to human health via drinking water, but the occurrence and nature of infection in wildlife is poorly characterized.

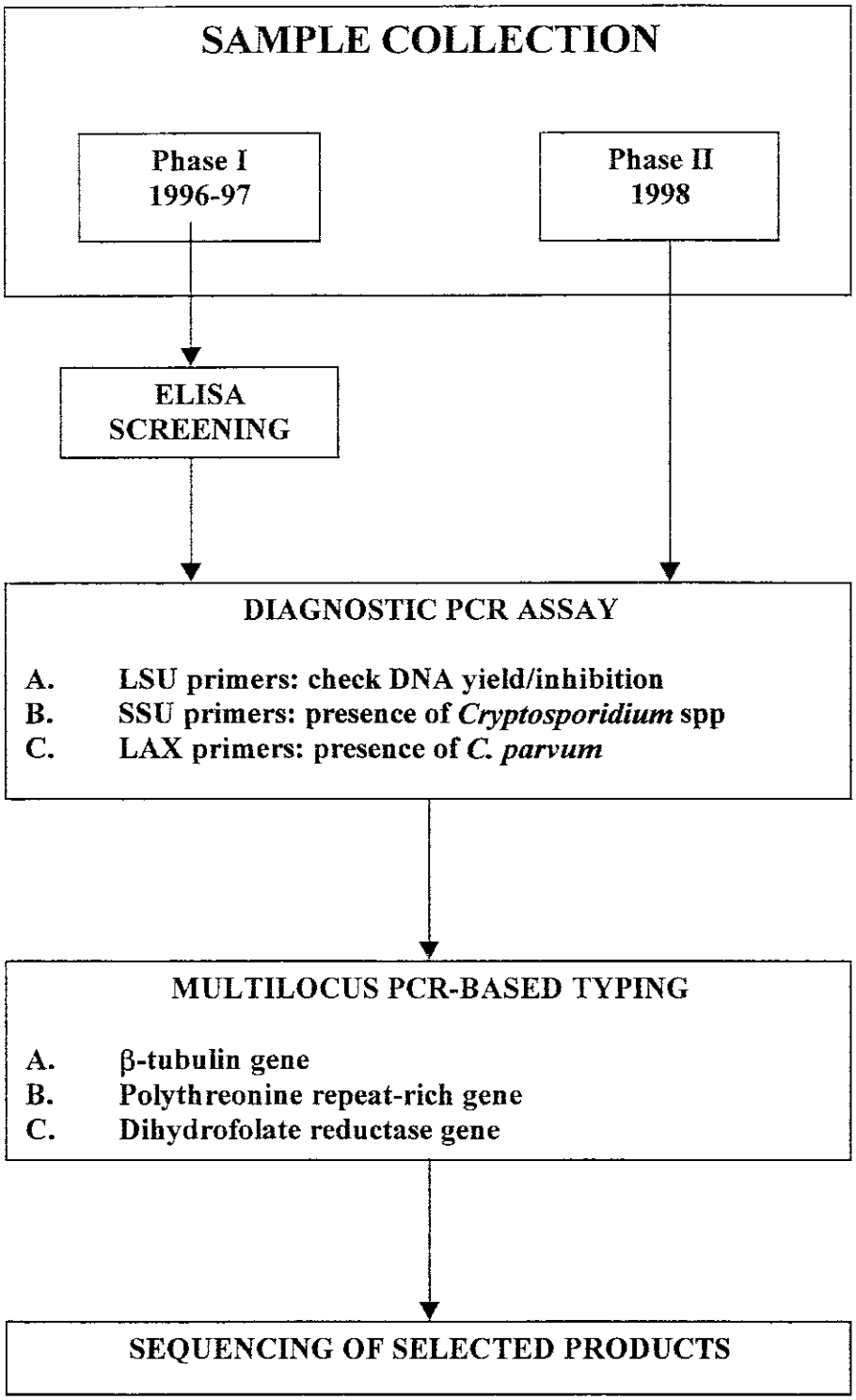


Figure 3-5: Schematic Representation of the Collection and Analysis of Wildlife Samples

b. Development of the PCR-based assay

Routine methods for the detection and diagnosis of *C. parvum* infection in feces are inadequate for the purposes of molecular epidemiology. Besides being relatively insensitive (Weber *et al.* 1991), they provide incomplete information with respect to the species of *Cryptosporidium* detected (i.e., limited specificity for *C. parvum* versus other *Cryptosporidium* species) and no means of differentiating isolates or strains (Weber *et al.* 1991). Because of these shortcomings, and in light of the fact that a PCR-based approach to the genetic characterization of isolates was the ultimate aim of the present study, it was determined that the development of a diagnostic PCR assay was required.

Diagnostic PCR assays for enteric bacteria have employed techniques for first culturing the microorganism of interest, thus providing a relatively pure, high concentration starting material for DNA extraction. This is very useful because direct extraction of DNA from fecal material is frequently associated with inhibition of the PCR (da Silva *et al.* 1999). Such an enrichment step is not applicable to *Cryptosporidium* because it cannot be cultured in the same fashion. Furthermore, protocols for the extraction of DNA from fecal material have not received extensive development and standardization, in contrast to blood and tissue samples.

Published protocols for diagnostic PCR of *Cryptosporidium* or related enteric protozoan pathogens (such as *Microsporidia* and *Entamoeba*) provided a starting point for the efforts described below, but these typically involved diarrheal samples known a priori to contain *Cryptosporidium* (da Silva *et al.* 1997; Katzwinkel-Wladarsch *et al.* 1994; Leng

et al. 1996). In contrast, a primary aim of the protocol developed here was the ability to detect *Cryptosporidium* DNA when oocysts are present in formed stool at low concentrations.

c. Diagnostic Primers

The three primer sets used in the diagnostic PCR assay provided a layered approach, (a) to screen for the presence/absence of PCR inhibitors, and (b) to detect *Cryptosporidium* from the mass of DNA in the samples. Formed stool contains an enormous array of microorganisms. Therefore, a primer set that could prime from a broad range of templates was very useful in determining whether the DNA preparation was free of PCR inibitors. Primers that appeared to be generic for large subunit rRNA genes (primer set LSU, Table 3-2) present in fecal samples from wildlife were identified; this set was selected for use in determining whether DNA preparations from fecal samples were successful in isolating and purifying DNA for the PCR. Reviews of published reports led to the selection and successful evaluation of two primer sets referred to herein as the SSU and LAX primers (Table 3-2) for use in the detection of *Cryptosporidium* (Johnson et al. 1995; Laxer et al. 1991). Both of these sets had favorable characteristics with respect to sensitivity, specificity, and reproducibility and have been used extensively in *Cryptosporidium*-diagnostic PCR applications. The SSU primers detect *Cryptosporidium* species other than *parvum* whereas the LAX primers reportedly detect only *parvum*. A visible product is expected to result from amplification with the generic LSU primers if sample DNA was prepared adequately. Next, the SSU primers would reveal whether

Table 3-2: Diagnostic Primer Sets used in this Study for Identification of *Cryptosporidium*

Part 1: Primer Sets

Primer Set	Target Gene	Primer Names/Sequences	Expected Product Size (bp)	Annealing Temperature[1] (°C)	$MgCl_2$[1] (mM)	References
LSU	Large submit rRNA	LSU1: 5'– GTAACTGCGAGTGAACAGGAA –3' LSU2: 5'– CCTCACGGTACTTGTTTGCT –3'	280	na/60	na/2.5	(this study)
SSU	Small submit RNA	CPB-DIAGF: 5'– AAGCTCGTAGTTGGATTTCTG –3' CPB-DIAGR: 5'– TAAGGTGCTGAAGGAGTAAGG –3'	435	55/60	3.5/2.5	Johnson *et al* 1995
LAX	Unknown	LAX1F: 5'– CCGAGTTTGATCCAAAAAGTTACGAA –3' LAX2R: 5'– TAGCTCCTCATATGCCTTATTGAGTA –3'	452	45/60	1.5/2.5	Laxer *et al* 1991

Note : Paired values indicate temperature or concentration specified in the original reference and in the present study, respectively.
na = not applicable

Part 2: DNA Probes For Use With The Above Primer Sets

Primer Set	Probe Information					Provided by
	Name	Source	Size	Vector	Reference	
SSU	p17-16	bovine – PCR product	1.7kb	pCRII	Tzipori 1994	Dr. G. Widmer, Tufts
LAX	pHCl	bovine – library	2.3kb	pUC18	Laxer 1991	NIH AIDS Reagent Program

Cryptosporidium spp. was present. Finally, the LAX primers would show if the *Cryptosporidium* species detected was *parvum*. Also, a perceived benefit of using both SSU and LAX primers was that each sample would be tested for the presence of *C. parvum* at least twice, increasing the odds of detecting low level infections.

Sequencing of SSU PCR products was planned as a means of characterizing presumptive *Cryptosporidium*-positive samples. Analysis of these sequences allows differentiation among *C. parvum*, *C. muris* and other species and may also be useful in identifying genotypes or strains within *C. parvum*.

d. Typing Primers

A number of genetic loci have been used successfully to discriminate between the human and bovine *C. parvum* genotypes. Primer sets were chosen that corresponded to three such targets for this analysis (Table 3-3). These targets were the beta-tubulin gene, a polythreonine-rich gene, and the dihydrofolate reductase gene, and are amplified by the B-TUB, POLY(T) and DHFR primer sets, respectively (Barnes *et al.* 1998; Caccio *et al.* 1997; Carraway *et al.* 1997; Spano *et al.* 1998; Vasquez *et al.* 1996; Widmer *et al.* 1998). The target sequences have been analyzed for the human and bovine genotypes, and the corresponding sequence polymorphisms have been documented; furthermore, each of these genes is present as a single copy (Spano *et al.* 1998; Vasquez *et al.* 1996; Widmer *et al.* 1998). For two of these loci, the beta-tubulin gene and the polythreonine-rich gene, multiple alleles have been detected and the two genotypes can be distinguished by restriction fragment length polymorphisms (which may thus avoid the labor and expense

Table 3-3: Typing Primer Sets used in this Study for Characterization of *Cryptosporidium*

Part 1: Primer Sets

Primer Set	Target Gene (Copy #)	Primer Names/Sequences	Expected Product Size (bp)	Annealing Temperature[1] (°C)	$MgCl_2$[1] (mM)	References
B-TUB	Beta-tubulin	BTUB5: 5'– GATTGGTGCTAAATTCTGGG –3' BTUB4: 5'– CCTGATCCTGTACCACCTCC –3'	478	52/60	n.s./2.5	Widmer *et al* 1998 Caccio *et al* 1999
POLY(T)	Polythreonine open reading frame of mucin-like glycoprotein	CRY44: 5'– CTCTTAATCCAATCATTACAAC –3' CRY39: 5'– GAGTCTAATAATAAACCACTG –3'	318	ns/52	n.s./2.5	Carraway *et al* 1997 Barnes *et al* 1998
DHFR	Dihydrofolate reductase-thymidylate synthase	DHFR1: 5'– TTGTGGCAGCTTCTGTTTTGA –3' DHFR2: 5'– GGATCGGCTTCATCTTGTGG –3'	232	68	2.5	Vasquez *et al* 1996

Note : 1. Paired values indicate temperature or concentration specified in the original reference and in the present study, respectively.
ns = not specified

Part 2: DNA Probes For Use With The Above Primer Sets

Primer Set	Probe Information				Provided by
	Name	Source	Size	Reference	
B-TUB	B-TUB3/4	bovine – PCR product	500 bp	na	Dr. G. Widmer, Tufts
POLY(T)	CRY44/37	bovine – PCR product	515 bp	na	Dr. G. Widmer, Tufts
DHFR	DHFR201	bovine – PCR product	200 bp	(this study)	na

Note: na = not applicable

involved in sequencing). Also, the B-TUB target contains an intron which may make it a hypervariable region, and useful for detecting intragenotypic or strain variations (Caccio *et al.* 1997; Widmer *et al.* 1998). The strategy called for all of the presumptive *Cryptosporidium*-positive samples plus ten PCR-negative samples to be amplified with all three primer sets (in separate reactions). The inclusion of a moderate number of PCR-negative samples was necessitated by the unknown specificities of the typing primers. Also, because the typing primers correspond to single copy targets in the *Cryptosporidium* genome, it was determined that an increased template concentration would be assessed.

2. MATERIALS AND METHODS

a. Methods Development: PCR Assay

A PCR-based assay was developed to detect the presence of *Cryptosporidium* in formed fecal specimens from wildlife. Development of the assay centered on two main aspects: (1) the preparation of sample DNA, and (2) the PCR-amplification. The preparation of sample DNA for use as template in the PCR required that DNA from a fecal sample be efficiently isolated and sufficiently purified. The major components are sample concentration, lysis, and purification. A review of the elements that were evaluated in the development of the protocol is presented in Table 3-4 (Arrowood and Sterling 1987; Ausubel *et al.* 1995; da Silva *et al.* 1997; Katzwinkel-Wladarsch *et al.* 1994; Leng *et al.* 1996; Ochert *et al.* 1994; Zhu *et al.* 1998). The recovery of DNA was assessed both

Table 3-4: Elements evaluated in the development of the DNA extraction protocol

ELEMENT	REFERENCES
Sample Concentration	
a. **filter through screen**	Weber *et al* 1997
b. PBS wash steps	daSilva *et al* 1997
c. formalin ethyl acetate (FEA)	Weber *et al* 1997
d. sucrose flotation	Arrowood and Sterling 1987
e. modified FEA	Weber *et al* 1997
Lysis Methods	
freeze-thaw	Leng *et al* 1996
chemical	
alkaline	Katzwinkel-Wladarsch *et al* 1994
SDS	Ochert *et al* 1994
DNAzol	Zhu *et al* 1998
chelex-100	Ochert *et al* 1994
boiling	Ochert *et al* 1994
homogenization, beadmill	daSilva *et al* 1997
heat treatment	Zhu *et al* 1998
Purification	
organic extractions	
phenol-chloroform	Ausubel 1995
chloroform	Ausubel 1995
amyl acetate	Dr. W. Wilfinger, MRC, pers. comm.
BMP	Dr. W. Wilfinger, MRC, pers. comm.
columns (chromatography)	
Geneclean (Bio101)	Katzwinkel-Wladarsch *et al* 1994
Qiaquick (Qiagen)	na
Proteinase K treatment	Ausubel 1995
Commercial Kits	
Pharmacia GenomicPrep	na
Pharmacia RapidPrep	daSilva *et al* 1997
Qiagen Qiamp	na

Note: Bold type indicates elements incorporated into the final protocol
na = not applicable

quantitatively (based on the total yield or concentration) and qualitatively (based on purity and size range). Typically, these evaluations were based on absorption spectroscopy or visualization on agarose gels using standard methods (Ausubel *et al.* 1995). Fecal material from deer samples collected in Phase I, with and without the addition of various spike levels, was used as the starting material. Spiking was performed by adding known concentrations of purified oocysts (isolate GCH1, derived from an experimentally infected calf, provided by Dr. G. Widmer, Tufts). Evaluations were also made using known concentrations of salmon sperm and/or *Cryptosporidium* DNA (previously extracted from isolate GCH1 or KSU-1). PCR itself was also used extensively to compare the yield and purity of DNA preparations. The resulting protocol (Table 3-5) involves: screening approximately one gram of fecal sample; suspension of the filtrate in a detergent lysing solution; bead mill homogenization and heat treatment; phenol-chloroform extraction; ethanol precipitation; and DNA solubilization and dilution.

The development of the PCR-amplification and product detection protocol (Table 3-6) involved both the optimization of reaction mix components and the selection and testing of appropriate primer sets. So-called "hot start" techniques have been shown to reduce mispriming and the generation of false products by preventing low temperature primer annealing (Moretti *et al.* 1998). Likewise, the use of bovine serum albumin (BSA) as an additive to the reaction mix has been shown to alleviate inhibition of the polymerase due to template impurities under certain circumstances (Kreader 1996). Hot start and BSA were incorporated into the protocol because both were found to be

Table 3-5: Protocol for the isolation and purification of DNA from stool samples obtained from wildlife

(Note: Processing of samples must be done in a designated area separate from the PCR set-up and post-PCR zones. Preferably, these should be different rooms.)

A. Wash/concentrate*

1. Mix approximately 1-1.5 grams of thawed fecal material with 4-6 ml ice cold PBS in a 15 ml tube; agitate sample using applicator sticks to create a slurry.
2. Pour slurry through funnel-screening device into 50 ml tube, washing with additional PBS to 50 ml; shake vigorously, centrifuge 5 min @ 500 g and decant supernatant.
3. Resuspend sediment with the use of a 2 ml transfer pipette and up to 1.5 ml additional PBS as needed; transfer to a 2 ml tube with screw top/o-ring and centrifuge 1 min @ 16,000 g (max speed).
4. Remove supernatant and record net weight of sediment.

B. Lysis

5. Suspend up to 600 mg of wet sediment with 1.1 ml DNAzol; fill tube w/ 2-3 grams 0.1 mm zirconium-silica beads.
6. Homogenize sample for 5 min at 4200 rpm using a Mini-Beadbeater; hold on ice.
7. Incubate in water bath for 10 min @ 90°C.
8. Pierce tube with 26G1/2 needle, piggyback on a new 1.5 ml tube inside of a 15 ml tube and centrifuge 3 min @ 2,000 g to recover lysate.

C. Purification and Precipitation

9. Transfer lysate to a 2 ml tube pre-filled with 1 ml Phenol:Chloroform:Isoamyl Alcohol (25:24:1).
10. Shake vigorously 20 sec, hold at room temp 3 min, centrifuge 7 min @ 16,000 g
11. Transfer lysate to 2 ml tubes pre-filled with 0.9 ml Chloroform:Isoamyl Alcohol (24:1).
12. Shake vigorously 20 sec, hold at room temp 3 min, centrifuge 7 min @ 16,000 g
13. Transfer 0.8 ml lysate to 1.5 ml tube pre-filled with 5 µl polyacryl carrier plus 0.4 ml (room temperature) 100% ethanol.
14. Invert 8-10 times, hold at room temp 3 min, centrifuge 5 min @ 5,000 g.

(continued on next page)

Table 3-5: Protocol for the isolation and purification of DNA from stool samples obtained from wildlife (continued)

D. Washing and Elution

15. Carefully remove supernatant by decanting; wash w/ 1 ml 95% ethanol, raising pellet by gentle vortexing or vigorously inverting tube.
16. Hold at least 5 min at room temperature, centrifuge 2 min @ 5,000 g.
17. Wash w/ 1 ml 95% ethanol as above.
18. Carefully remove ethanol by pipetting, allow pellet to air dry for approximately 10 min.
19. Add 100 µl 8 mM NaOH, incubate at 65°C for 5 min, solubilize w/ gentle vortexing as needed, and hold on ice.
20. Centrifuge 10 min @ 16,000 g to remove insoluble material.
21. Transfer supernatant to new tube containing 10 µl 0.1M HEPES (final pH ~8).
22. Create 0.1X dilution with molecular biology grade H_2O.

* Note that certain small mammal samples (see text for details) were subjected to alternate wash/concentration steps in order to remove the fecal material from SAF preservative and to permit these samples to be stored prior to further processing as per this protocol.

Materials

15 ml tube (Falcon)
50 ml tube (Falcon)
applicator sticks (Fisher)
funnel-screening device (Meridien Contrate)
transfer pipette (Fisher)
2 ml tube (screw top, O-ring) (Biospec)
DNAzol (MRC)
0.1 mm zirconium-silica beads (Biospec)
Mini-Beadbeater (Biospec)
26G1/2 needle
Phenol:Chloroform:Isoamyl Alcohol, 25:24:1 (Gibco Life)
polyacryl carrier (MRC)
sterile (molecular biology grade) H_2O (5prime3prime)

Table 3-6: Protocol for PCR-amplification of DNA from stool samples

A. In area designated for PCR set-up, assemble the following reagents and associated materials including dedicated pipettors and filtered pipette tips.

Reagent	Volume (µl)	Final Concentration (in 50 µl Reaction)	Master Mix Component
Sterile H_20	14.75	---	✓
10x PCR Buffer	5	1x	✓
DNTP mix (d-A,C,G,T-TP)	4	0.2 mM each	✓
$MgCl_2$, 25 mM	5	2.5 mM	✓
BSA, 4mg/ml	3	1.2 µg per reaction	✓
Forward Primer	4	0.4 mM	✓
Reverse Primer	4	0.4 mM	✓
Taq DNA polymerase	0.25	1.25 units per reaction	✓
"Make-up" H_20 (sterile)	0-9	---	---
gDNA Template	1-10	Variable	---
Total	50	---	(40 µl)

B. Include the following negative controls: one "master mix" control receiving sterile H_20 before addition of *Cryptosporidium* genomic DNA to any reactions; "blanks" (at least one per 12 sample reactions) receiving sterile H_20 during template additions. Also include a positive control consisting of gDNA at a low but reliable level (e.g. 1 picogram).

C. Prepare fresh Master Mix of all the above reagents except template and make-up H_20, adding reagents in the order listed. Scale volumes appropriately for the number of reactions planned. Mix tube by inversion and then centrifuge briefly.

D. Label 0.5 ml reaction tubes and arrange in rack(s).

E. Add sterile "make-up" H_20 to tubes as needed Add approximately 60 µl mineral oil to each tube.

F. Add 40 µl master mix to each tube.

G. Relocate to designated area for handling DNA samples.

H. Mix thoroughly by gently tapping tubes containing DNA template and add 1 µl template to each tube, taking care to avoid cross-contamination.

I. Centrifuge the reaction tubes briefly at low speed (e.g. 30 sec @ 1000 g) to collect the contents at the bottom of the tube.

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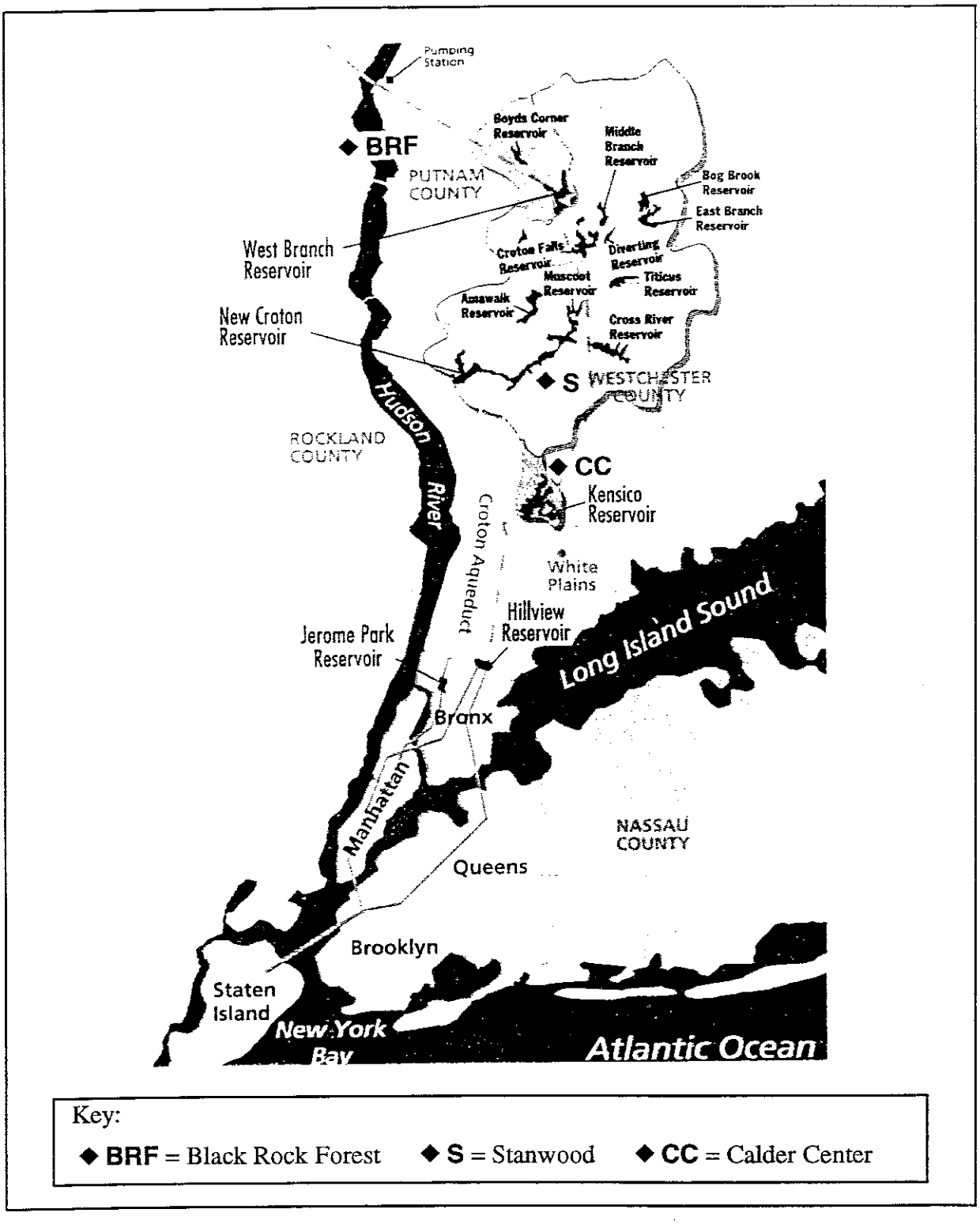
dedicated to each phase. Positive and negative controls were included in every experiment; one out of every twelve reaction tubes consisted of a "blank" in which template was omitted

b. Wildlife Survey: Sample Collection and ELISA Screening

Wildlife fecal samples were collected from the following three sites (Figure 3-6):

- Black Rock Forest. The Black Rock Forest is a 1500 hectare research station located in Cornwall, New York, 50 miles north of New York City in close proximity to the Hudson River. Black Rock Forest is maintained by a consortium of institutions including Columbia University.
- Louis Calder Center of Fordham University. The Calder Center is a biological field station located in Westchester County in Armonk, New York, within the local watershed of the Kensico reservoir.
- Stanwood/New Croton Reservoir. A permit (Appendix C) was obtained from the NYC DEP allowing access to city-owned forested lands buffering the New Croton Reservoir in Westchester County, New York for the purpose of conducting this study. Samples were collected from the southern portion of the reservoir, concentrating on the areas in the vicinity of the Stanwood community in the town of New Bedford.

The primary focus was on the collection of fecal samples from white-tailed deer. Deer samples were collected on the basis of their morphology and in relation to other indicators such as tracks and visual observation. Black Rock Forest Manager personnel



Modified from NYCDEP "BW&SO/AJ" 1996

Figure 3-6: Wildlife Survey Sampling Sites

provided training in this regard and assisted in the collection of deer samples. Trapping of animals was not considered feasible within the context of this survey, so fresh fecal material was collected from the ground and then frozen for subsequent analysis. Fecal samples from other mammals were collected when circumstances allowed this or were provided by Black Rock Forest and Calder Center personnel, as noted in Tables 3-7 and 3-8. Samples provided by Dr. Thomas J. Daniels of the Calder Center were from animals that were trapped as part of an ongoing study of the role of small mammals in the transmission of Lyme disease; these samples were stored in SAF preservative and held at 4°C for up to one week prior to performance of the ELISA assay. Note that individual samples may not be from unique animals. In fact, sampling of deer during Phase II was designed to survey small populations on successive visits in a relatively short time frame.

Phase I Samples. All of the Phase I samples were screened for the presence of *Cryptosporidium* antigen using a commercial ELISA kit (ProSpecT/*Cryptosporidium*, Alexon Inc, Mountain View, CA) following the manufacturer's protocol. A total of 238 samples were collected and assayed in duplicate during Phase I; six samples were considered positive after consistently meeting the manufacturer's optical density criteria. The collection and analysis of Phase I samples is summarized in the following table:

Table 3-7: Summary of Phase I Sampling and ELISA Results

Site	Time Period	Host[1]	N	ELISA-positive
Black Rock Forest	Jan-Feb 1996	White-tailed deer	106	0
		Coyote[2]	3	0
		Gray fox[2]	1	0
	May-June 1996	White-tailed deer	32	2
		Other	1	1
	March 1997	White-tailed deer	13	0
Stanwood	October 1996	White-tailed deer	17	0
	March 1997	White-tailed deer	27	0
Calder Center	Sept-Oct 1996	White-tailed deer	13	0
		White-footed mouse[3]	8	0
		Eastern Chipmunk[3]	9	0
		Gray Squirrel[3]	1	0
		Striped Skunk[3]	2	1
		Raccoon[3]	5	2

Notes: 1. Common/Latin names of host species are as follows:

White-tailed deer / *O virginianus*
Gray Fox / *Urocyon cinereoargenteus*
Gray Squirrel / *Sciuris carolinensis*
Raccoon / *Procyon lotor*
Coyote / *Canis latrans*
Chipmunk / *Tamias striatus*
Skunk / *Mephitis mephitis*
White-footed mouse / *Peromyscus leucopus*

2. Sample(s) collected by Mr. John Brady
3. Sample(s) collected by Dr. Thomas J. Daniels

After the ELISA screening of the Calder Center samples, approximately 12 (including the three that were ELISA-positive) were washed with PBS to remove the SAF preservative and were subsequently stored at 4°C in 2.5% potassium dichromate prior to preparation of DNA for use in the PCR assay (as per Table 3-6)

Phase II Samples. During Phase II, sampling efforts focused mainly on the Westchester County sites. A total of 103 samples were collected; 91 of these were from white-tailed deer (Table 3-8), six were from muskrats, and six were from samples for which the host could not be identified. These samples were held at -20°C until analysis.

Table 3-8: Summary of Phase II Sampling						
Site	Black Rock Forest		Stanwood		Calder Center	
Dates	6/05/98	6/27/98	6/04/98 6/10/98 6/25/98	7/02/98 7/10/98	6/10/98 7/02/98	7/10/98
Type	Deer	Unknown	Deer	Unknown	Deer	Muskrat[1,2]
N	18	2	54	4	19	6

Notes: 1. Samples were collected under the guidance of Dr. Thomas J. Daniels;
2. Common/Latin names: Muskrat / *Ondatra zibethicus*

c. Diagnostic PCR

Sample preparation and PCR-amplification: Phase II samples. All of the Phase II samples (n = 103, # 501-603) were screened for the presence of *Cryptosporidium* as follows. DNA was extracted using the standard DNA isolation and purification protocol (Table 3-5). All samples were initially subjected to PCR-amplification with the LSU primer set using 0.5% of the total DNA extracted per sample (5 µl @ 0.1x dilution), following the standard DNA PCR protocol (Table 3-6). As described previously, this was done to confirm the adequacy of each sample DNA preparation to serve as a template for the PCR reaction. Following electrophoresis, PCR products consisting of single clear bands of the expected size (280 bp) were detected for 87 of the samples. The remaining 16 samples were characterized by faint products of the expected size (n = 13) or by an absence of product (n = 3). The DNA concentration of these 16 sub-optimum samples was assessed visually by gel electrophoresis as follows. Known quantities of salmon sperm DNA alongside 15% (15 µl) of the 1x sample DNA preparations was loaded on an agarose gel pre-stained with ethidium bromide and photographed under ultraviolet light. For comparison, the DNA concentrations of seven samples that amplified successfully were also assessed in the same manner. In general, the yields of both the sub-optimum samples and the successfully amplified samples were comparable.

This suggested that inhibition was a more likely explanation than insufficient DNA in the reactions. Therefore, the amount of template used in further PCR reactions performed with the 16 sub-optimum samples was reduced 60% (to 2 µl @ 0.1x). For four samples with low DNA yields, two template levels were employed: both the reduced and standard amounts (i.e. 2 and 5 µl @ 0.1x). All of the Phase II samples were then subjected to PCR-amplification with the SSU and LAX primers as per the standard protocol using the template levels just described.

Sample preparation and PCR amplification: Phase I samples. PCR screening of selected Phase I samples for the presence of *Cryptosporidium* was performed in two parts. The first part included (a) four ELISA-positive small mammal samples (#'s 4229, 4250, 4254, and AX1), (b) three ELISA-negative small mammal samples (#'s 4244, 4230, and 4252), and (c) two samples that served as positive controls for the DNA preparation process. The process controls were: GCH1P, an unprocessed fecal sample from an experimentally infected calf at the Tufts School of Veterinary Medicine (provided by Dr. G. Widmer), and #447, an ELISA-negative deer sample spiked with 10^5 oocysts (previously purified from isolate GCH1) per gram. Samples were processed by a preliminary version of the standard DNA isolation and purification protocol, which omitted the organic extraction steps and appended a purification step using column chromatography (Qiagen Qiaquick). PCR-amplification was performed using the SSU and LAX primers; the LSU primers were not used with these samples. The PCR protocol was as specified in the standard protocol, except that hot start was performed by an alternate means (Ampliwax, PE

Biosystems). A single template level – approximately 5% of the total extract yielded from 100-300 mg (wet weight) of filtered fecal material – was used in all reactions.

The second part of the PCR-screening of Phase I samples included both deer (n = 8) and small mammal (n = 13) samples, six of which were also processed in part one. Two of the deer samples were ELISA-positive (#'s C1 and C2) as were four of the small mammal samples (#'s 4229, 4250, 4254, and AX1). GCH1P (described above) was again included as a process control. Four additional phase II deer samples were also processed with this batch to assess reproducibility (#'s 501, 506, 524, and 598). DNA from these samples was prepared using the standard protocol with the exception that, for four samples, the starting material had been previously subjected to an ethyl-acetate concentration procedure. The DNA yields were assessed by gel elctrophoresis as previously described. PCR-amplifications were performed as per the standard protocol. PCR-amplifications were first performed using the LSU primers with 10-100 nanograms of sample DNA per reaction to assess the appropriate template levels. PCR-amplifications were subsequently performed with the SSU and LAX primers with two levels of template (in general, approximately 5-10 and 10-100 nanograms per reaction).

Detection of PCR-amplified Products / Southern Analysis. Following PCR-amplification, 10 µl of each sample was electrophoresed on a 3.5% NuSieve (FMC Corp.) agarose gel. The gels were then stained, viewed under ultraviolet light and photographed. DNA was transferred from the gels via blotting onto nylon filters using standard methods. Southern analysis of the PCR products from the SSU and LAX amplifications was performed using

the appropriate DNA probes (Table 3-2). Overnight hybridizations were performed at 65°C and the stringency of the final post-hybridization wash was 0.2 SSC @ 65°C. Records of the hybridization signals were made by autoradiography.

d. Typing PCR

Samples. A total of 43 wildlife samples were selected from among both phases of the wildlife survey (n = 341) for further characterization. Among these samples, 33 were presumptive PCR-positives and 10 were PCR-negative. The calf sample (GCH1P) was also included as a positive control. DNA had been extracted previously (as described above), but new dilutions were prepared for all applicable samples for use as PCR template.

PCR-amplification and detection of products. In order to determine the appropriate template concentrations for the 43 samples selected for typing analysis, a review of the previous PCR-amplifications was made. Additionally, a PCR amplification with the LSU primer set was repeated for most of the samples to assess the feasibility of using higher levels of template in order to increase the chances of detecting single copy sequences. PCR-amplifications with the typing primers were subsequently performed using two established levels for each sample following the standard protocol. The detection of products by visualization on gels and by Southern analysis was performed as described above for the diagnostic PCR. The DNA probes used in these Southern analyses are described in Table 3-2.

e. Cloning and Sequencing

SSU/PCR products. SSU/PCR products from ten of the wildlife samples were selected for cloning and sequencing. Products were cloned into the PCRII-TOPO plasmid vector using the TOPO cloning kit (Invitrogen). Eight of the ten samples were cloned and sequenced successfully. The nucleotide sequences of these clones were determined by the dideoxy-chain termination method. The M13R and T7 primers were used to provide the sequence from both DNA strands. An additional SSU product, obtained from PCR-amplification of a sample from a human patient diagnosed with *Cryptosporidium* infection at the Columbia Presbyterian Medical Center (New York) (see Appendices D and E) was also similarly cloned and sequenced.

PCR Products from Typing primers. Products from selected PCR-amplifications with the B-TUB, POLY(T), and DHFR typing primers were cloned and sequenced. Methods were identical to those used for the SSU PCR products, except that DHFR and POLY(T) clones were only sequenced on one stand (using the T7 primer). Cloning and sequencing were successful in one of two attempts for B-TUB products, in one of four attempts for POLY(T) products, and in both attempts for DHFR products.

3. RESULTS

a. Diagnostic PCR

33 positive wildlife samples were detected from among a total of 125 samples (22 from Phase I and 103 from Phase II) tested using the diagnostic PCR assay (see Table 3-9). As described previously, a presumptive *Cryptosporidium*-positive sample was defined as one that was positive with either the SSU or LAX primers following Southern analysis. Positive controls, using either purified genomic *C. parvum* DNA or DNA from the process control sample GCH1P, resulted in strong gel bands and intense hybridization signals in all instances. No products were detected in lanes containing negative controls. Positive wildlife samples generally displayed less hybridization signal than the positive controls. In general, the hybridization signal was consistent with the intensity of the bands visualized on the stained gels (see Figure 3-7). Longer exposures were required during audioradiography to detect weak signals from certain samples. Samples exhibiting very weak signal or signal intensities that did not correspond to the staining of the gel bands were designated borderline positives as noted in Table 3-9. Such results where the signal is very low but the PCR product is present in a large amount suggest that the sequences of the PCR product and probe differ considerably. For the reactions using the LAX primers, no false bands (DNA products other than those of the expected size) were detected on the stained gels. In addition, visual detection of positive PCR products on the stained gel was in complete agreement with the results of Southern analysis. In other words, the autoradiography did not reveal additional positive samples. For the reactions using the SSU primers, false bands were frequently detected especially

Table 3-9a: Results of the PCR-amplifications with the diagnostic and typing primer sets as detected by direct visualization on stained gels (G) and Southern analysis (S)

Host	Site[1]	I.D.[2]	Diagnostic Primers				Typing Primers					
			SSU		LAX		B-TUB		POLY(T)		DHFR	
			G	S	G	S	G	S	G	S	G	S
Calf	–	GCH1P	●	●	●	●	●	●	●	●	●	●
Deer	S	506	○	●	○	○	○	○	●	●	○	○
Deer	S	524	●	●	○	○	○	○	○	○	○	○
Deer	S	538	○	○	●	●	○	○	○	○	○	○
Deer	S	540	◑	◑	○	○	◑	○	○	○	○	○
Deer	B	553	◑	●	○	○	○	○	○	○	○	○
Deer	S	563	●	●	○	○	◑	◑	○	○	○	○
Deer	S	569	●	●	○	○	◑	○	◑	○	○	○
Deer	S	570	●	●	○	○	◑	○	○	○	○	○
Deer	S	571	●	●	○	○	nd	nd	nd	nd	nd	nd
Deer	C	576	○	●	○	○	○	○	○	○	○	○
Deer	S	578	○	◑	○	○	○	○	○	○	○	○
Deer	S	586	●	●	○	○	○	◑	○	○	○	○
Deer	C	598	●	●	●	●	◑	○	○	○	○	○
Deer	B	UR1	●	●	○	○	○	○	●	●	○	○
Deer	S	J6	○	○	●	●	●	◑	●	●	○	○
Deer	B	C2	○	○	●	●	◑	○	●	●	●	●
Deer	S	(501)	○	○	○	○	○	○	●	●	○	○
Deer	B	(A3)	○	○	○	○	◑	○	●	●	○	○
Deer	S	(548)	○	○	○	○	◑	○	○	○	○	○
Deer	S	(580)	○	○	○	○	◑	○	○	○	○	○
Deer	C	(594)	○	○	○	○	◑	○	○	○	○	○

Key: ● = positive; ◑ = borderline positive; ○ = negative; Shaded backgrounds indicate products that were sequenced. nd = not determined

Notes: 1. Sites: S=Stanwood; B=Black Rock Forest; C=Calder Center

2. Parentheses indicate samples that were judged negative by the diagnostic PCR.

Table 3-9b: Results of the PCR-amplifications with the diagnostic and typing primer sets as detected by direct visualization on stained gels (G) and Southern analysis (S)

Host	Site[1]	I.D.[2]	Diagnostic Primers				Typing Primers					
			SSU		LAX		B-TUB		POLY(T)		DHFR	
			G	S	G	S	G	S	G	S	G	S
Unknown	B	AX1	○	●	○	○	○	○	○	○	○	○
Unknown	B	513	○	◑	○	○	○	○	○	○	○	○
Unknown	S	567	◑	●	○	○	◑	◑	○	○	○	○
Skunk	C	4229	○	●	○	○	◑	●	●	●	○	○
Raccoon	C	4250	○	●	○	○	○	○	○	○	○	○
Mouse	C	4227	●	●	○	○	●	●	●	●	●	●
Mouse	C	4252	○	◑	○	○	○	○	●	◑	●	●
Chipmunk	C	4234	●	●	○	○	●	●	○	○	●	●
Chipmunk	C	4237	●	●	○	○	●	●	●	●	●	●
Chipmunk	C	4244	○	◑	○	○	○	○	○	○	○	○
Chipmunk	C	4247	●	●	●	●	○	○	●	●	●	●
Muskrat	C	589	●	●	○	○	◑	●	●	○	○	○
Muskrat	C	590	●	●	○	○	◑	◑	○	◑	○	○
Muskrat	C	591	●	●	●	●	◑	◑	●	◑	●	●
Muskrat	C	592	●	●	○	○	◑	○	○	○	○	○
Muskrat	C	593	●	●	○	○	○	○	○	○	○	○
Muskrat	C	603	●	●	○	○	◑	○	●	◑	●	●
Grey Fox	B	(GFQ)	○	○	○	○	○	○	○	○	○	○
Chipmunk	C	(4246)	○	○	○	○	○	○	○	○	○	○
Unknown	S	(549)	○	○	○	○	○	○	○	○	○	○
Raccoon	C	(4230)	○	○	○	○	○	○	○	○	◑	◑
Raccoon	C	(4254)	○	○	○	○	○	○	○	○	○	○

Key: ● = positive; ◑ = borderline positive; ○ = negative; Shaded backgrounds indicate products that were sequenced.

Notes: 1. Sites: S=Stanwood; B=Black Rock Forest; C=Calder Center

2. Parentheses indicate samples that were judged negative by the diagnostic PCR.

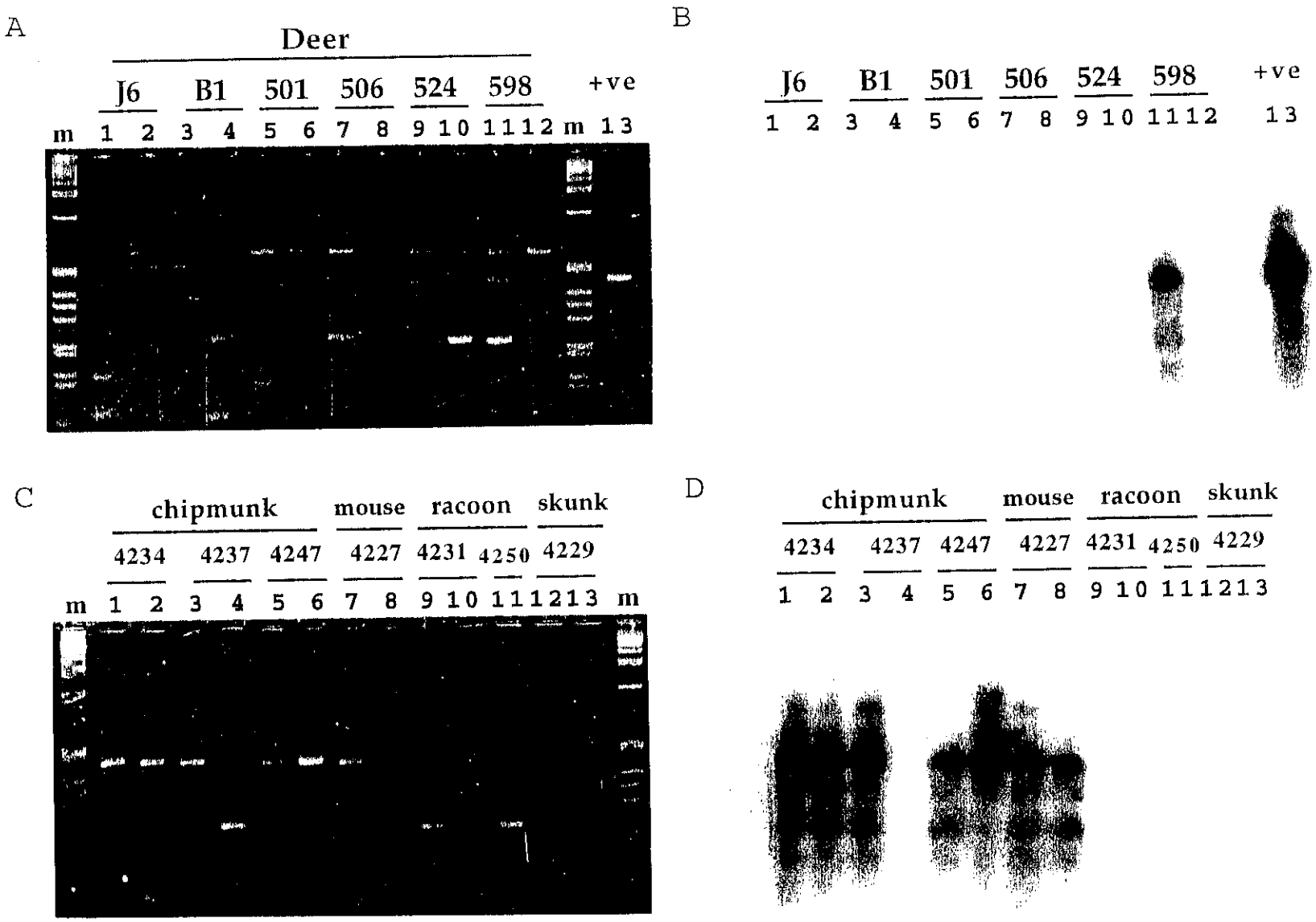


Figure 3-7: PCR amplification products from the SSU diagnostic primer set with subsets of deer (panels A & B) and small mammal (panels C & D) samples as visualized directly on agarose gels (panels A & C) and by Southern analysis with the SSU probe (panels B & D).
Two template concentrations were used for each sample (except 4250). The left and right lanes of each pair are the high and low concentrations, respectively. +ve is a PCR positive control of 2 pg of GCH1 DNA. Lanes marked m are size markers.

among deer samples, suggesting non-specific amplification of SSU rDNA from organisms other than *Cryptosporidium* (e.g. lanes 1-10, Figure 3-7). Southern analysis revealed positive samples in addition to those apparent from the stained gels. In all, of the 30 samples designated as PCR-positive with the SSU primers, nine were not apparent from the gels, demonstrating the increased sensitivity afforded by the Southern analysis with the SSU primers and probe.

Among the deer samples, 16 out of 99 tested positive (Table 3-9a). Of these, 13 were positive only with the SSU primer sets. This led to the hypothesis that the deer may be primarily infected by *C. muris*. Among the small mammal samples, 17 were positive out of a total of 26 samples tested (Table 3-9b). Of these, two samples (chipmunk #4247 and muskrat #591) were positive with both primer sets while the remainder was detected only with the SSU primers. Again, it was hypothesized that *C. muris* might account for most of these infections; most of the small mammal positives (all except the raccoon and the three unknowns) were from rodent hosts. Note that successful amplification was partly a function of the level of template used in the PCR, and that for certain samples (e.g deer #506 and #524, Figure 3-7) amplification was inconsistent, presumably reflecting low levels of target DNA and/or variable levels of inhibiting substances in the template preparations.

The results of the diagnostic PCR assay did not correlate well with the ELISA results for the Phase I samples. Of the six ELISA-positives, four were also PCR-positive (#'s AX1, 4229, 4250 and C2) while two were PCR-negative (#'s 4254 and C1). Failure to amplify

these two samples with the PCR could not be explained by inhibition. Among the 16 ELISA-negative samples, six were PCR-positive, consistent with the premise that the PCR assay is more sensitive.

b. Sequencing Analysis of SSU Products

The human isolate, CP01, resulted in the amplification of a SSU product with a sequence that was identical to the typical human genotype Type A rDNA sequence, as confirmed by BLAST analysis. Eight SSU products from the wildlife survey were sequenced, four from deer and two each from the chipmunk and muskrat samples. For three of the deer-derived SSU sequences, a high degree of sequence similarity to *C. parvum* was found. The sequence for #598, the one deer sample that was also LAX-positive, was identical to the bovine *C. parvum* Type A SSU sequence. This finding was consistent with the view that the LAX primers are specific for *C. parvum*. Two other deer SSU sequences (#569 and #586) were >98% identical to each other and produced very similar results upon BLAST analysis (see Appendix F for an example of output). Alignments with the entire lengths of these products were only found with *C. parvum* sequences. This includes the one example of a *C. wrairi* SSU rDNA sequence available, which is interpreted as a *C. parvum* strain as described previously (Xiao *et al.* 1998). For both deer #569 and #586, 97% identity with the *C. wrairi* sequence and 95-96% identity with the *C. parvum* sequences resulted. The next most significant matches, which did not align within the 5' hypervariable region, were with *C. baileyi*, *C. muris*, and *C. serpentis* sequences. These were followed by matches with sequences from other protozoa. The interpretation of these data is that the SSU sequences from deer # 569 and #586 most likely originated

from *C. parvum*. The remaining deer sequence, from sample #540, was not a *Cryptosporidium* sequence. The sequenced product was longer (476 bp vs. 435 bp expected), and BLAST analysis showed significant alignments corresponding to the SSU genes from other microorganisms (e.g. *Cercomonas*); however, no sequence in GenBank™ produced significant alignment over the whole length of the cloned product. For this sample, the SSU product visible on the gel appeared to be slightly larger than the target size and the same was true for the cloned product fragment. However, it had been designated as a borderline positive because a single faint product of the correct size (as per the positive controls) was evident on the autoradiograph.

Analysis of the four rodent-derived SSU sequences revealed one (chipmunk #4237) that was identical to the bovine genotype Type B SSU gene. For the other chipmunk (#4247), the SSU sequence was identical to the sequence from deer #586. The muskrat sequences were also closely related to #586/#4247; muskrat #603 differed from these by only one base pair (3' of the hypervariable region) while muskrat #591 contained some additional differences. BLAST searches for the muskrat SSU sequences were essentially as described above for deer #569 and #586. Again, the evidence suggests that these novel SSU sequences most likely originated from *C. parvum*.

Multiple sequence alignments (Figure 3-8) were carried out for the hypervariable region of the SSU to gauge the relationship of the wildlife isolates with each other, and with the established *C. parvum* isolates. Additionally, alignments were made with four unique *C. parvum* strains (canine, pig, mouse and koala) designated as "animal, non-bovine" that

Notes:
* Reference sequence for non-human genotype sequences
1. Bold sequence descriptor indicates sequence resulting from this study
2. GenBank accession numbers are indicated in parentheses where applicable
3. reference = Le Blancq *et al*, 1997
4. reference = Morgan *et al*, 1998
5. reference = Morgan *et al*, 1997

Figure 3-8: Multiple sequence alignments within the hyper-variable region (nt 633-696) of the *Cryptosporidium* SSU rDNA gene (SSUV) between wildlife sequences and reference sequences

were obtained from searches of the GenBank™ database and from published reports (Morgan *et al.* 1997; Morgan *et al.* 1998). Most of the heterogeneity among the sequences is confined to two short subregions (corresponding to nucleotides 636-654 and 686-694, using numbering as per bovine sequence L16996). All three of the novel wildlife sequences detected appear to be more similar to each other than to any of the reference sequences. The total number of mismatches and/or gaps in this region with respect to the bovine Type A reference sequence was compared. In order of similarity, the new wildlife sequences (with six or seven differences each) fall between *wrairi* (four differences) and the four non-bovine animal sequences (nine to twelve differences each). These regions were also the sites of heterogeneities between Type A and Type B units. As discussed in Part One of this chapter, such intraorganismal heterogeneities complicate interpretation. For example, one of the wildlife sequences (e.g. #591) could be the companion Type B unit to the Type A sequences amplified from a different sample, perhaps #586/4247/603. Additionally, the possibility of sequence heterogeneities arising from PCR artifacts (i.e. poor fidelity of the *Taq* polymerase) must be considered; this may be addressed by repeating the PCR-amplification, cloning, and sequencing for any suspect sample. Nonetheless, this analysis does strongly suggest that these novel wildlife isolates should be considered *C. parvum* variants. Contrary to earlier indications from the absence of PCR products with the LAX primers, none of the sequenced products appeared to originate from *C. muris*.

c. Typing PCR

Results with the three sets of typing primers were variable (see Table 3-9). Reactions using GCH1P, the process control calf sample, worked well in all instances, but results with wildlife samples were inconsistent, as described below.

DHFR. Among the 33 *Cryptosporidium*-positive wildlife samples examined, only eight produced the expected DHFR product; all but one of these (deer #C2) occurred in rodent samples (see Table 3-9b and Figure 3-9). In addition, one borderline positive DHFR reaction was found among the *Cryptosporidium*-negative samples (raccoon #4230), suggesting that the primer set is not 100% specific or that the diagnostic assay failed to correctly identify this sample as *Cryptosporidium*-positive. (Note that negative PCR controls were always negative in these experiments and that rigorous technique was used to prevent spillover and other sources of false positives.) The fact that many of the samples failed to amplify may reflect a lower sensitivity with these primers than with the SSU set. Sequence for the DHFR product was obtained from two samples, mouse #4227 and muskrat #603. In both instances, the sequences were identical to each other and different from the reference bovine genotype sequence by only one nucleotide, at one of the eight positions where the human and bovine genotypes were previously shown to differ (see Figure 3-10) (Vasquez *et al.* 1996).

POLY(T). Among the 33 *Cryptosporidium*-positive wildlife samples examined, 12 produced the expected POLY(T) product (Table 3-9). Four of the deer and eight of the others (all rodents) were positive; however, four of the rodent positives were judged

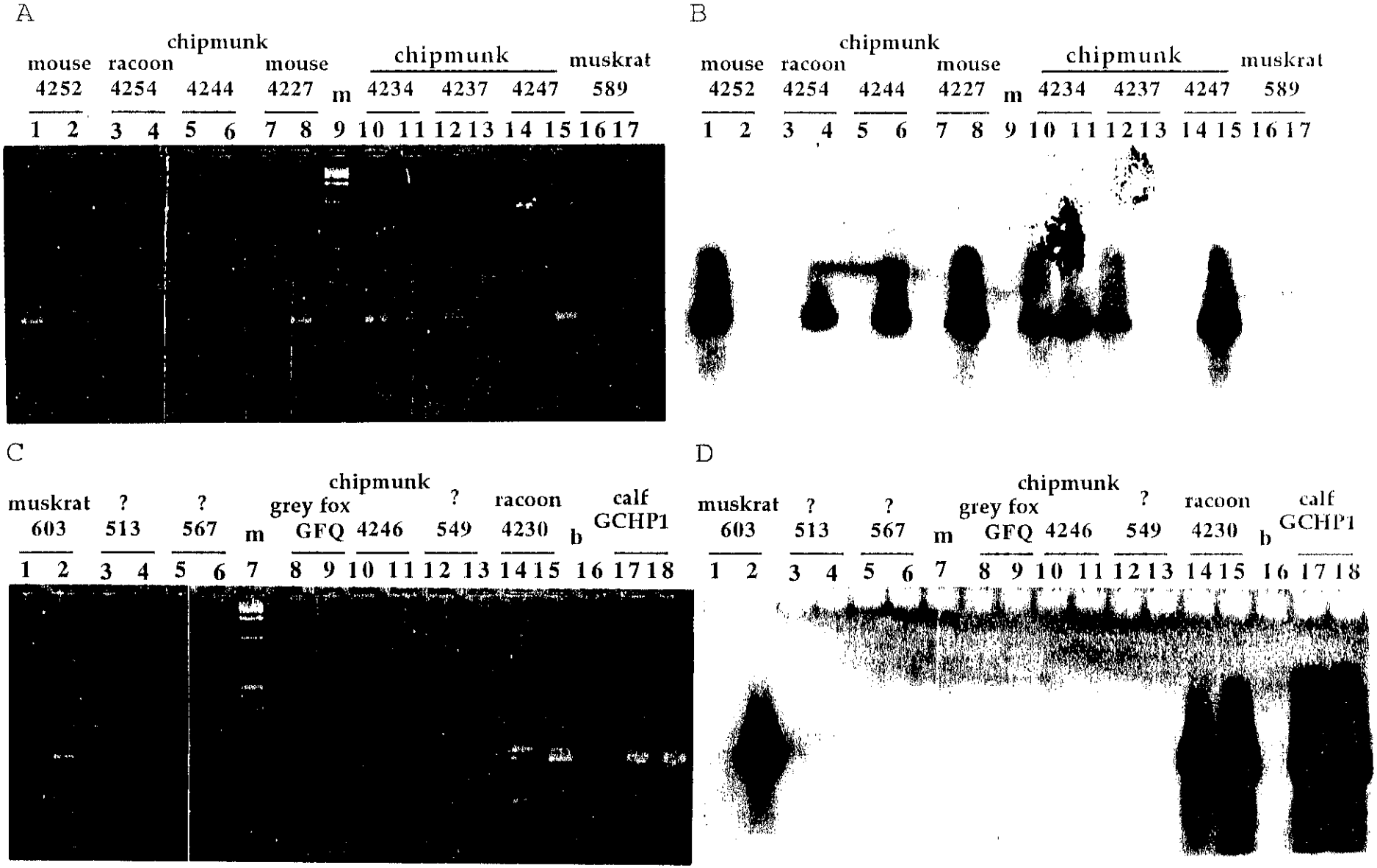


Figure 3-9: PCR amplification products from the DHFR typing primer set with subsets of small mammal samples visualized directly on agarose gels (panels A & C) and by Southern analysis with the DHFR probe (panels B & D).

Two template concentrations were used for each sample. The left and right lanes of each pair are the high and low concentrations, respectively. Lanes marked m are size markers. Lane marked b is an empty lane. "?" indicates unknown host species.

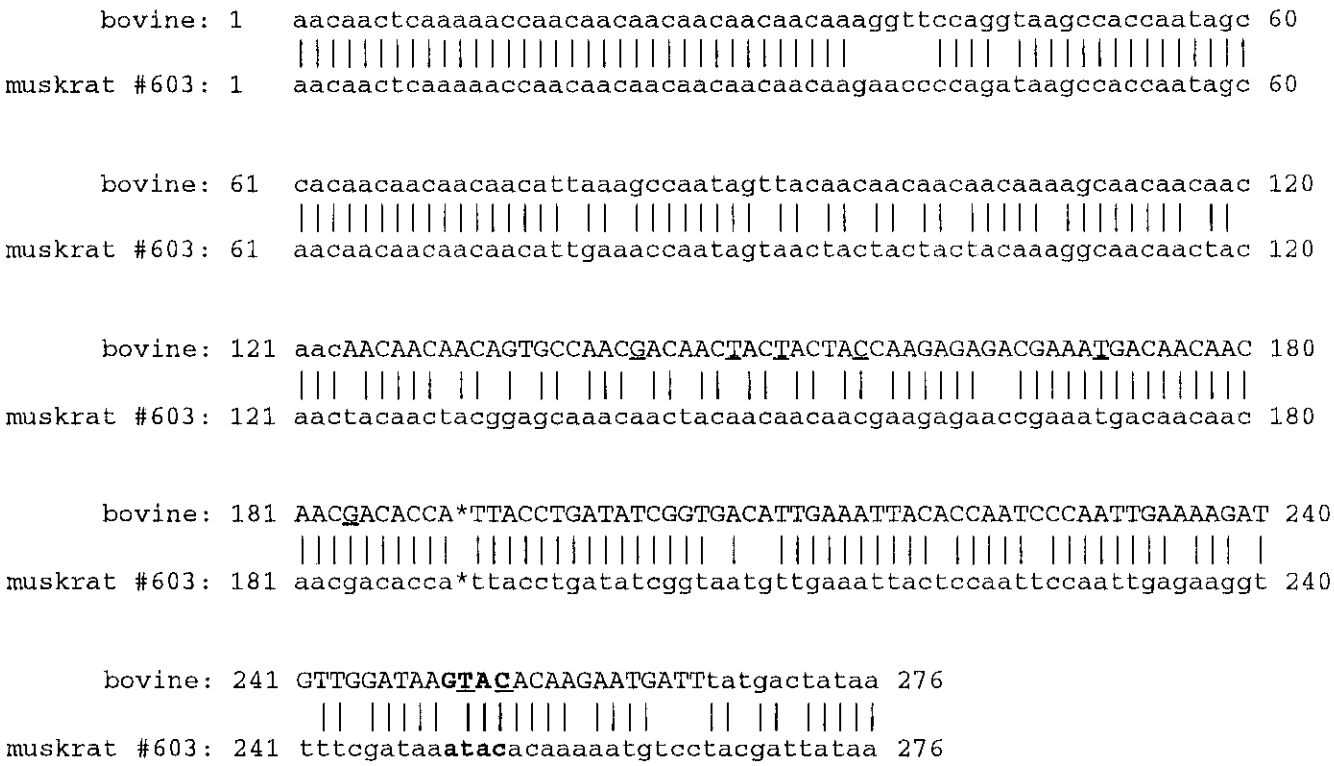
```
        bovine: 174 TTGTGGCAGCTTCTGTTTTGAGTAGAGGAATAGGAATTAACGGACAATTACCTTGGT 230
.muskrat/mouse:   1 ............................................................ 60
         human:  59 ......................C..T..G........................... 115

        bovine: 231 CTATATCTGAGGATTTGAAATTTTTTTCAAAGATAACTAGTAATAATTGTGACTCGAATA 290
muskrat/mouse:  61 ............................................................ 120
         human: 116.....................................A......A............. 175

        bovine: 291 AGAAGAATGCACTAATTATGGGAAGAAAAACATGGGATTCAATTGGAAGAAGACCTCTTA 350
muskrat/mouse: 121 ............................................................ 180
         human: 176 ............................................................ 235

        bovine: 351 AAAATAGAAAAATAGTCGTTATTTCCTCTAGCTTACCACAAGATGAAGCCGATCC 405
muskrat/mouse: 181 ............T..................................... 232
         human: 236 .........T...T............C..................... 290
```

Notes: Numbering of reference sequences corresponds to GenBank submissions (accession numbers: bovine = U41365; human = U41366). Locations of primers is indicated by underlining.

Figure 3-10: Alignment of the DHFR PCR product sequences from muskrat sample #603 and mouse sample #4227 and the corresponding sequences from the bovine and human *C. parvum* genotypes

borderline on the basis of ambiguous results in the Southern analysis. Among the *Cryptosporidium*-negative wildlife samples, two (deer #501 and #A3) were also positive, raising questions regarding the specificity of this primer set. One of the POLY(T) products, a borderline positive muskrat (#603) was successfully sequenced. Sequencing revealed an actual product length of 319 bp (versus 318 bp expected). BLAST analysis of the nucleotide sequence showed significant alignment over the entire sequence only with *C. parvum*. However, only 84 percent identity was seen in alignments with the two available reference (bovine genotype) *C. parvum* sequences. (Results were similar when a BLAST analysis of the translated amino acid sequence was performed.) This may explain the ambiguous signal detected for this sample in the Southern analysis. It is unclear whether the sequence obtained was actually from *Cryptosporidium* or from some unidentified organism. For a 143 bp segment of the POLY(T) product, 10 base pair differences were reported between the human and bovine genotypes (Widmer 1998) whereas 24 differences were detected between the muskrat and bovine genotype sequences (see Figure 3-11). It is possible that this degree of heterogeneity is consistent with the identification of a novel strain of *C. parvum* but the significance of these findings is currently unclear. Sequence analysis of other PCR products from these samples will provide clarification.

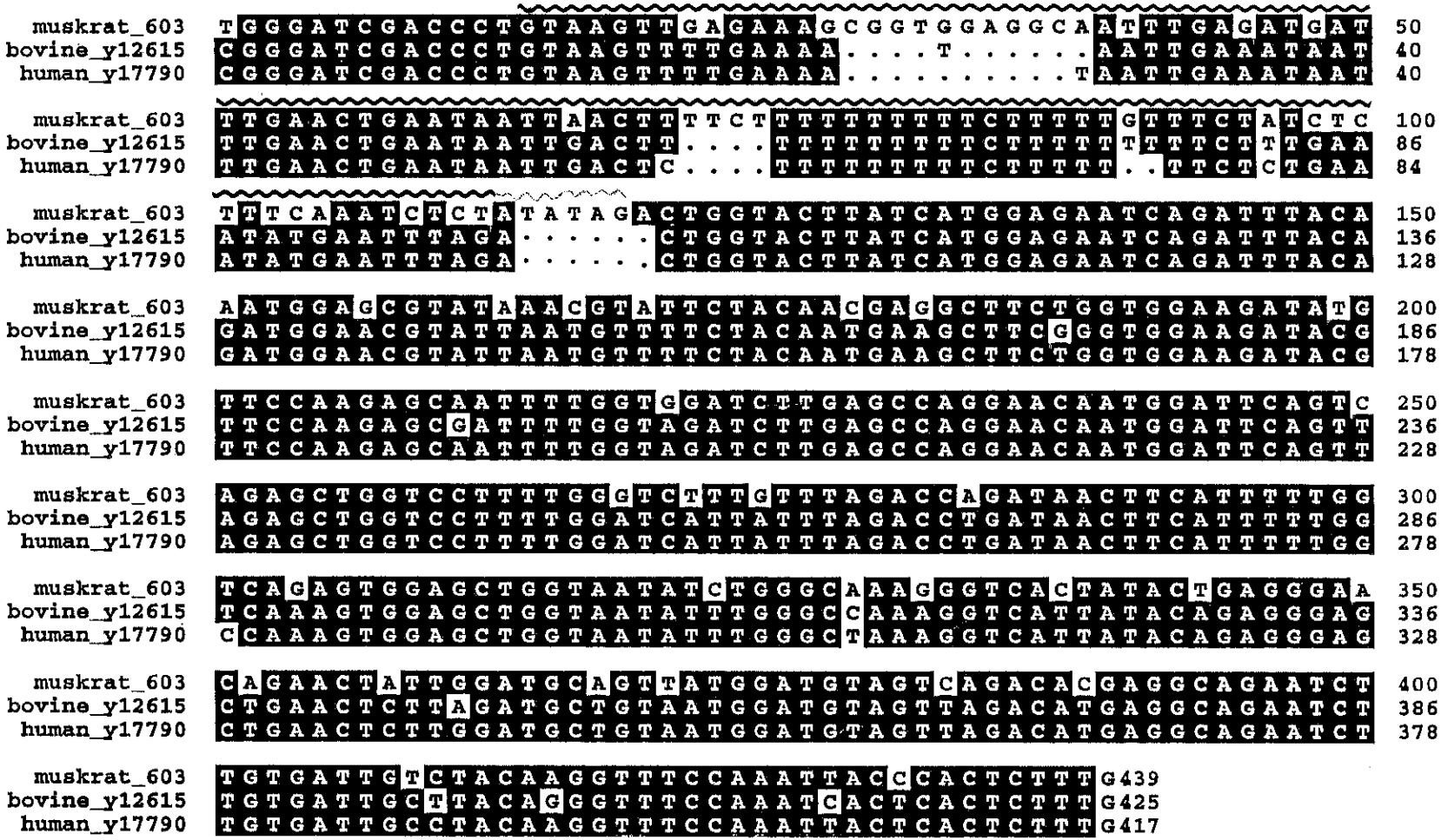
B-TUB. Interpretation of the B-TUB reactions was complicated by the frequent appearance of false products. In particular, there were numerous occurrences of products that were within 5% of the expected size; frequently these hybridized with the probe DNA. As the beta-tubulin sequence is highly conserved across species, some



Notes: Region of the reference sequence (GCH1 isolate) shown in uppercase was previously characterized with respect to differences between human and bovine genotypes (Widmer 1998); underlined nucleotides indicate positions of such differences (locations are approximate in some instances due to discrepencies in the orginal). Asterisks denote position of a trinucleotide (CCA) insertion present in the human genotype. Bolded region at nucleotides 250-253 indicates the location of a RsaI restriction site that was found to be present in bovine isolates. Primer sequences are not included.

Figure 3-11: Alignment of POLY(T) PCR product sequence from muskrat sample #603 with reference sequence from the *C. parvum* bovine genotype

hybridization signal from PCR products from unrelated species can be expected. This led to the designation of numerous borderline positives because of uncertainty regarding the degree of size heterogeneity that may actually be present in this target among *Cryptosporidium* species and strains. Only 5 of the B-TUB reactions were considered clear positives; these all occurred among rodent samples (Table 3-9b). Cloning and sequencing were attempted with one of these clear positives (mouse #4227) but was not successful. Sequence was determined for one product (muskrat #603) which appeared larger than the expected size and did not hybridize clearly. The length of the cloned fragment was 498 bp, versus 480 bp expected. This size discrepancy is due primarily to differences in the size of the intron (see Figure 3-12). BLAST analyses showed that the most significant alignments to sequences in the GenBank™ database were with the *C. parvum* beta-tubulin gene, followed by beta-tubulin sequences from other microorganisms. Relative to the bovine and human reference sequences, the muskrat sequence had 86% and 89% identity, respectively, compared with 98% identity between the established genotypes. Once again, interpretation of this finding is difficult; it may have originated from an unknown organism or it may be bona fide, perhaps reflecting an actual *Cryptosporidium* strain difference.



Notes: Location of the intron is indicated by the wavy line. Primer sequences have been deleted. Numbering corresponds to the GenBank submissions (accession numbers as shown). The bovine sequence shown reflects a 6bp correction at the 5' end of the intron, as per Widmer *et al.* 1998.

Figure 3-12: Alignment of the B-TUB PCR product from muskrat sample #603 and the corresponding sequences from the bovine and human *C. parvum* genotypes

4. DISCUSSION

Molecular tools for the detection and characterization of *Cryptosporidium* were successfully developed and applied in this study. These were used to demonstrate the existence of a sylvatic transmission cycle involving deer and other mammalian hosts. This cycle appears to involve the bovine genotype of *C. parvum* as well as at least one additional novel strain closely resembling but distinct from the established *parvum* genotypes and other recently identified strains. Previously, unique strains have only been reported as isolates from a single host species (Morgan *et al.* 1997; Morgan and Thompson 1998), whereas this study detected a unique strain among three different mammalian hosts (deer, muskrat and chipmunk).

The detection of *C. parvum* among both deer and rodent hosts demonstrates that wildlife serve as a potential source of infectious oocysts within New York City's Westchester County watersheds. Under the auspices of the New York City Department of Environmental Protection, a pathogen survey of wildlife in the upstate Catskill-Delaware watersheds is ongoing; as of January 1997, *Cryptosporidium* oocysts had been detected in the feces of one white-footed mouse (New York City Department of Environmental Protection 1997).

Overall, the relevance of the present findings to human health is unclear. The isolates detected were apparently associated with relatively low levels of oocyst excretion and were generally recovered from asymptomatic hosts (i.e. formed stools). The extent to

which oocysts present in wildlife feces enter the water is not known. The survival and movement of oocysts in agricultural and sylvatic settings has received only limited study (Walker *et al.* 1998). In this regard, the finding of *Cryptosporidium* infections in muskrats may be of some significance as these are aquatic rodents that spend much of their time in water. Coincidentally, there have been no previous reports of *Cryptosporidium* infection in either muskrats or skunks, with the exception of a recent report of infection in muskrats in Poland (Sinski *et al.* 1998). Also, the present study represents the first time that *C. parvum* isolates from chipmunks or muskrats have been analyzed genetically.

Larger wild mammals such as white-tailed deer may provide a significant reservoir of infection because of the substantial quantity of droppings they produce (Sturdee *et al.* 1999). In Phase II of the present study, 12 % (12/98) of the deer samples tested was positive by the PCR assay. While this study was not designed to accurately characterize prevalence, one can appreciate the role that deer may play in terms of transmission among sylvatic mammals and in terms of water quality impacts. Also, regardless of the prevalence or concentration of *Cryptosporidium* oocysts in wildlife species, it is clear that there will be a contribution to the environmental load (Sturdee *et al.* 1999). Even in the complete absence of anthroponotic or agricultural sources, watersheds preserved in a "pristine" state may not be adequately protected in this regard.

Cryptosporidium infection was first reported in white-tailed deer in a captive herd in the state of Georgia, among a small sample of neonatal fawns and their mothers; most

infections were asymptomatic (Fayer *et al.* 1996). Recently, the first study of *Cryptosporidium* infection in wild white-tailed deer was reported (Rickard *et al.* 1999); the prevalence of infection was determined by microscopic methods. Among fawns (age < 6 months) presented at a wildlife center in Virginia, 9% (3/34) were infected. Deer aged 6 months to > 7 years were surveyed throughout Mississippi and were found to have an overall prevalence of *Cryptosporidium* infection of 5% (18/360). From these data and the present findings in New York, widespread carriage of *Cryptosporidium* infection in this host species seems apparent.

Based on morphometric analysis, the previous reports of infections in deer were designated as *C. parvum* (Fayer *et al.* 1996; Rickard *et al.* 1999). In addition, for one of the captive deer isolates, the SSU rRNA gene sequence was recently submitted to GenBank™ (Accession # AF093494) and was identical to the *C. parvum* bovine genotype. In the present study, two of the positive isolates, one from a deer and one from a chipmunk, were also identical within the amplified regions to the bovine genotype rDNA unit. Therefore, these samples most likely represent *C. parvum* strains within the established bovine genotype which are capable of infecting humans. However, the relevance of the additional novel strains detected in the present study to human health is not clear. Little is known about the extent of strain variability within *Cryptosporidium*, or the associated differences in infectivity and virulence. Published reports of novel *C. parvum* strains other than *wrairi* have only recently surfaced and are few in number, coming mostly from an Australian-led group (Morgan *et al.* 1997; Morgan *et al.* 1998). In all cases, the primary evidence for regarding these as genetically distinct (non-human,

non-bovine) strains comes from analysis of the SSU hypervariable region that was also examined in the present study. Novel *C. parvum* strains were isolated from pigs (three in Australia and one in Switzerland), a single koala and in a group of five wild mice (see Figure 3-9) (Morgan *et al.* 1997; Morgan *et al.* 1998). Furthermore, all three were described as distinct host adapted "genotypes". Additionally, *Cryptosporidium* from domestic Australian cats was shown to be genetically very distinct from all other *Cryptosporidium* species or isolates; these oocysts were also significantly smaller in size (Sargent *et al.* 1998). As mentioned earlier, these findings support the designation of *C. felis* as a separate species or strain. Analysis of the novel genotypes at other genetic loci has been very limited. Morgan *et al* examined the acetyl-Coenzyme A synthetase gene. They found that the mouse isolates grouped separately from the human and bovine genotypes while the pig isolate grouped with the bovine; interestingly, the koala isolate and three of the four pig isolates did not amplify in their PCR analysis (Morgan *et al.* 1998). This variability illustrates the critical importance of examining multiple loci when performing a genotyping analysis.

Morgan *et al* interpreted their findings as evidence for conserved host-adapted *Cryptosporidium* genotypes, some of which may be distinct species and/or host-specific (Morgan *et al.* 1998). However, this view of host-adapted or host-specific strains has been challenged by both the present findings of a novel strain in three different host species and by other recent findings. First, *C. felis* which was previously only identified in domestic cats, has now been confirmed in repeated isolates from a cow, based on both morphometric and SSU sequencing analyses (Bornay-Llinares *et al.* 1999). Second,

researchers at the Centers for Disease Control and Prevention have prepared a report on the identification of two novel *Cryptosporidium* strains in immunodeficient patients (Pieniazek *et al.* 1999). One of these is the canine strain mentioned earlier, which the CDC has found in domestic dogs and in persons with AIDS. The other strain identified was actually *C. felis*, and like the canine strain has been recovered from multiple patients and in repeated samples. In addition, yet another novel strain, resembling the pig isolate in its SSU sequence, was also recently recovered from an AIDS patient (Dr. N. Pieniazek, CDC, personal communication). These findings suggest that while the human and bovine genotypes may account for the majority of human *Cryptosporidium* infections, this picture is far from complete. Also, isolates may have been mischaracterized if targets were examined that do not discriminate non-human, non-bovine strains. Risks from species or strains of *Cryptosporidium* found in mammalian hosts but not fitting into the two established *C. parvum* genotypes may not be summarily dismissed as irrelevant. Instead, the emergence of zoonotic strains in humans deserves close attention (Tzipori and Griffiths 1998).

The power of the molecular tools used in this study was evidenced by the extent of useful information that was derived despite incomplete success with some of the component strategies. For example, the LAX primer set did not perform as anticipated and, in retrospect, was not useful. In developing the PCR assay, the LAX primers were as sensitive as the SSU primers, but did not perform as well with the wildlife samples. It is possible that this primer set and its corresponding target sequence are actually restricted to a subgroup of *C. parvum*. Including this set in the diagnostic approach provided little

additional information, and the inferences that were made regarding the presence of *C. muris* (in samples that were SSU-positive but LAX-negative) were contradicted by the sequencing results.

IV. IMPLICATIONS AND FUTURE DIRECTIONS

Cryptosporidium is a major public health problem, as demonstrated by its significant prevalence and negative health impacts in immunocompromised populations and also by the number of waterborne outbreaks that have occurred. In the absence of useful treatments, prevention is essential but is complicated by a lack of knowledge concerning the transmission routes associated with human infections.

The work presented in this dissertation was aimed at addressing this shortcoming and has provided some new approaches and insights to the study of *Cryptosporidium* epidemiology. The risk assessment and modeling work showed the importance of placing case surveillance for cryptosporidiosis within the context of HIV/AIDS surveillance. More importantly, the premise of low level endemic transmission via tap water was supported. It was shown that such transmission could contribute substantially to background levels of case rates and infection in a manner consistent with observations from surveillance programs.

Also, the disproportionate numbers of cases occurring in immunocompromised subpopulations is consistent with common source exposures, given the mechanisms by which cases are detected and recorded. Awareness of the inadequacies of case surveillance activities has led to calls for useful revisions and new approaches (Frost and Craun 1998; Griffiths and Morris 1999; Proctor *et al.* 1998). Furthermore, basic questions regarding the definition of safe drinking water are now receiving discussion and debate (Franco 1997; Gerba *et al.* 1996; Miller 1998).

Another avenue of investigation that is receiving increased attention is the use of serologic surveys. Serological testing for *Cryptosporidium* antibodies has been proposed using serum collected for other purposes such as blood lead screening in targeted groups such as children (Griffiths and Morris 1999). Such surveys could provide indications of recent or longer-term exposure and/or infection, depending on the methods used, and could be used as part of ongoing surveillance programs or in specific studies (Frost *et al.* 1997). Significantly, this approach has the potential to assess the effect of water supply between communities or changes in water treatment within a population (Griffiths and Morris 1999).

Previous exposure and protective immunity should be considered when an assessment of the risks associated with *Cryptosporidium* (or other pathogens) is performed. Previous exposure may affect the susceptibility to infection or the severity of illness associated with infection. Recently published findings from a study with subjects who were re-challenged with *C. parvum* provide the first relevant data in this regard (Chappell *et al.* 1999). One year after the original challenge (DuPont *et al.* 1995), subjects (all of whom had developed anti-*Cryptosporidium* serum IgG) exhibited increased resistance to infection, as evidenced by an ID_{50} that was approximately 20-fold higher than in the original, immunologically naïve, cohort (Chappell *et al.* 1999). It has been further suggested that the risk of an outbreak may vary inversely with the level of endemic exposure (Frost and Craun 1998). This hypothesis is compatible with the observation

that most outbreaks have been detected in filtered or groundwater systems (Frost and Craun 1998).

Additional evidence from volunteer studies is emerging regarding heterogeneity in the virulence of different *Cryptosporidium* strains (Okhuysen *et al.* 1997). In addition to the (IOWA) strain used in the original and re-challenge volunteer studies, preliminary evidence from two additional strains, TAMU and UCP, is now available. Interestingly, these two strains appear to vary by a factor of 100 in their ID_{50}'s – 500 oocysts for UCP and 6 for TAMU – while bracketing the (revised) ID_{50} of 90 oocysts for the IOWA strain. For the infectivity parameter used in the model developed in Chapter II, the implication from these data is that a wider confidence interval but a similar central tendency value would be appropriate. Of further note, the data from the TAMU strain support the premise that exposure to a single oocyst, even among healthy persons, is associated with a genuine risk of infection (i.e. there is no evidence for a threshold dose level).

Before turning away from the subject of risk assessment, consideration should be given here to the evidence for the two major genotypes, human and bovine. Because human genotype *parvum* may be prevalent in drinking water and in the environment generally, information on the relative infectivity of this genotype would certainly be desirable. However, because of an inability to produce infection in experimental animals, such data may be lacking for some time. Furthermore, the likelihood that such information would substantially alter the model's basic findings is perhaps small.

Genotyping is a powerful tool that has only recently been introduced to the study of *Cryptosporidium* epidemiology. Previously established molecular typing approaches have been of great use in analyzing the epidemiology of other infectious agents. A major drawback in applying these techniques to the study of *Cryptosporidium* infection is that they generally require large amounts of purified oocysts (Widmer 1998). PCR-based approaches overcome this shortcoming while providing the added benefit of finer resolution. Indeed, the advent of PCR-based strain resolution in the study of *Cryptosporidium* presents an entirely new level of analysis for this pathogen. The capacity to discriminate the major *Cryptosporidium* genotypes (or species) associated with human infections, plus the ability to discriminate within and beyond them, promises to add significantly to our knowledge of the natural history of *Cryptosporidium*.

Understanding genetic heterogeneity should provide important insights into phenotypic characteristics such as infectivity, virulence, and drug sensitivity (Widmer 1998). Along these lines, genotypic analyses of isolates from the human volunteer studies are being performed (Widmer 1998). Such work may also prove useful in evaluating the zoonotic potential of the novel strains detected in the present study and elsewhere, and in explaining the inability of human genotype *parvum* isolates to produce infections outside primates or even in cell culture. In sum, by relating genotypes to phenotypes, genotyping may usefully focus on relevant markers in the future (Maslow *et al.* 1993).

In order for these efforts to provide maximally useful insights into *Cryptosporidium* epidemiology, it is critical that genotyping is (1) firmly grounded and properly

cular and phylogenetic senses, and (2) properly integrated with

logic studies and approaches.

st point, *Cryptosporidium* from various hosts and many isolates

characterized via multilocus analysis (Spano *et al.* 1998). An

d classification is needed, as this will provide insights to

ost ranges and zoonotic potential (Morgan *et al.* 1998). To this end,

l RNA genes will provide a useful avenue, and the work presented

presence of distinct rDNA types is of obvious utility. It is important

er loci in *Cryptosporidium* continues, in conjunction with analysis of

rovide an integrated and useful basis for distinguishing isolates. To

g is being planned by the U.S. Centers for Disease Control and

Environmental Protection Agency to address the current state of

notyping (Dr. A. A. Lal, CDC, personal communication).

g is best used to support and augment classic epidemiologic

w *et al.* 1993). For example, when applied in conjunction with

s of outbreak investigation (e.g. case control analyses), genotyping may

ying the source of contamination or factors responsible for its spread.

s of the current study was to compare the species and genotype of

isolates from wildlife in New York City's watersheds with those found

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Appendix A:

A Framework for Assessing the Risks of Waterborne *Cryptosporidium*

J.F. Perz, F.K. Ennever, and S.M. Le Blancq

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A Framework For Assessing The Risks Of Waterborne *Cryptosporidium*

Joseph F. Perz[1], Fanny K. Ennever[1], and Sylvie M. Le Blancq[1,2]
Division of Environmental Health Sciences[1]
Columbia University School of Public Health
Center for Environmental Research and Conservation[2]
Columbia University
New York, NY

BACKGROUND

While waterborne transmission is well established in community outbreaks of cryptosporidiosis, the significance of low levels of occurrence of this parasite in non-outbreak settings remains unclear. Uncertainty regarding tapwater's contribution to endemic cryptosporidiosis relative to other exposures has complicated the formulation of regulations and guidelines for protecting public health. This paper describes a risk assessment framework for examining the potential role of tapwater in the transmission of *Cryptosporidium* infection. The plausibility of such a role is considered, along with the question of whether the risk assessment predictions can be reconciled with reported levels of disease.

Population-based risk assessment models have been used to estimate the risks of *Cryptosporidium* infection from tapwater consumption, the desired levels and benefits of improved water treatment, and also to examine the validity of the risk assessment approach itself (1). Two analyses have explicitly addressed the issue of reconciliation of *Cryptosporidium* risk assessments with actual disease rates; these considered the outbreak in Milwaukee (2) and endemic disease in the Pacific Northwest (3). The investigators concluded that the risk assessment approach was generally supported in the outbreak situation, but not for the analysis of the endemic disease, which described much lower rates of illness evident in the population than predicted by their model.

It is important to relate predicted infection rates to clinical outcomes and the likelihood that an infection will result in a case report to the appropriate health authority. Not all infections with *C. parvum* produce symptoms (4, 5). Symptomatic infections are marked by diarrhea that is sometimes severe yet self-limiting in immunocompetent persons, while in persons with Acquired Immune Deficiency Syndrome (AIDS), diarrhea due to *C. parvum* infection tends to be chronic and life-threatening (5). Diagnosis of *Cryptosporidium* infection requires stool testing, meaning that only those infections which result in illness and physician contact have the potential to come to the attention of the appropriate health agency (6). Therefore, risk assessments ought to consider persons with AIDS separately. AIDS represents the late clinical stage of infection with the human immunodeficiency virus (HIV). The current surveillance definition of AIDS includes persons who have HIV infection and either CD4+ T lymphocyte (CD4) counts (cells/μl) lower than 200 or the diagnosis of a specific opportunistic infection, such as cryptosporidiosis of greater than one month's duration. Persons with AIDS have patent immune deficiency. Nonetheless, they do not seem to be inherently more susceptible to all enteric pathogens (7). Consistent with other AIDS-related opportunistic infections, cryptosporidiosis in persons with HIV infection is manifested chiefly as a late stage infection mainly evident in advanced AIDS (CD4 counts < 100) (7). Although reactivation of latent infections (e.g. as CD4 counts decline) may play a role in the appearance of

cryptosporidiosis (8), waterborne outbreaks have indicated the significance of recent exposures in the acquisition of cryptosporidiosis in persons with AIDS (9).

Risks of infection from consumption of tapwater

In order to generate central tendency estimates of the rates and numbers of annual infections resulting from exposure to low levels of *Cryptosporidium* in tapwater, this analysis adopted the general approach that has been employed for microbial risk assessment. Consistent with the biology of *C. parvum*, exposure to a single oocyst is assumed to be associated with risk of infection in humans. Dose-response data were used to evaluate the probability that a single oocyst may initiate a **detectable** infection (4, 10). This probability was derived by applying an exponential ("single hit") model. The estimate of the single organism infectivity was reported as 1:239 (0.0042), with 95% confidence limits of 1:132 to 1:454 (10). The model for relating exposure to infection is as follows:

$$I_j = Pop_j \times \{1 - \exp(-dose_j \times r_j)\} = Pop_j \times \{1 - \exp(-Q_j \times C \times r_j)\}$$

At low doses (i.e. when the value z in exp(-z) is less than 0.1), the exponential model may be replaced with a simple linear relationship:

$$I_j = Pop_j \times Q_j \times C \times r_j$$

where:

I	=	calculated number of infections / year
Pop	=	population of exposed subgroup
j	=	subgroup
Q	=	mean annual tapwater intake (L/yr)
C	=	relevant *C. parvum* concentration (organisms/L)
r	=	single organism infectivity (infection/organism)

Data from a U.S. national survey in 1977-78 (11) provide an average daily tapwater intake of 1.13 liters per day. This baseline intake was reduced by 40%, to account for the preparation of hot beverages and foods which are expected to render *Cryptosporidium* oocysts non-infective. Additional adjustments to reflect greater tapwater avoidance since the time of the survey were not made for the general population. However, based on evidence that persons with AIDS may exhibit significant avoidance of tapwater (9), that group's average intake was estimated to be further reduced by one-third.

For risk assessment purposes, an estimate of the long term (e.g. annual) average occurrence in tapwater of viable *C. parvum* oocysts which are both infective and pathogenic to humans is required; in other words, the concentration that is relevant to public health. Currently, no methodology exists which can be reliably applied to measure this concentration. Most of the measurement data presented in the U.S. thus far suggest average concentrations of one or fewer *Cryptosporidium* oocysts per 100 liters (12-14). Such concentrations are near or below most "best case" detection limits because, frequently, only a fraction of the sample is analyzed and recovery efficiencies may be less than 5-10%. Measured concentrations may approximate the relevant concentration, if it is assumed that recovery inefficiencies are balanced by over-counting of non-viable, non-pathogenic organisms (1, 10). Two concentrations, one oocyst per 1,000 liters and one oocyst per 100 liters, were selected for this analysis with the preliminary assumption that these may represent relevant *C. parvum* concentrations.

In order to perform a population-based risk assessment, three factors that influence the appropriateness of utilizing the derived infectivity estimate for different segments of the general population must be considered (4, 10). First, data were derived from a single strain of *C. parvum*. Currently there is no information available on the infectivity of other *C. parvum* strains, and the assumption must be that the strain utilized is typical. The two remaining factors are host-related; study subjects were healthy adults, and study candidates who were seropositive for *Cryptosporidium* were excluded. Due to the absence of supporting data, no adjustment was made to the derived infectivity for the non-AIDS population; it was assumed that persons who may be more susceptible than the volunteers are balanced by persons who may be less susceptible for reasons such as partial immunity from prior exposure.

Since persons with AIDS do not clear the parasite, colonization will be more likely to be followed by infection and illness. This is important because the infectivity derived from the volunteer study may underestimate the probability of colonization, as relatively high levels of oocyst excretion are required for clinical detection. This analysis assumed a three-fold higher infectivity (0.0126) for persons with AIDS.

Outcomes following infection

The sequence of events leading from infection to case reporting was cast as a series of conditional probabilities, as follows:

$$R_j = I_j \times pD_j \times pM_j \times pV_j \times pO_j \times pC_j \times pR_j$$

where:

R	=	calculated number of reported cases / year	
pD	=	p(D | I)	= p(diarrheal illness | infection)
pM	=	p(M | D)	= p(moderate-severe illness | D)
pV	=	p(V | M)	= p(physician visit | M)
pO	=	p(O | V)	= p(ova & parasite exam | V)
pC	=	p(C | O)	= p(*Cryptosporidium* test | O)
pR	=	p(R | C)	= p(case detection and reporting | C)

Quantitative estimates of the probability of each of the component events were derived from recommended approaches to the investigation and treatment of diarrheal illnesses and from epidemiologic and clinical studies of *Cryptosporidium* infection. An abbreviated description of the derivation of these probabilities is presented in the following sections.

p(diarrhea | infection), pD. *Cryptosporidium* infection may or may not result in symptomatic illness (4, 5). This analysis uses the definition of diarrheal illness from the human volunteer study (three or more unformed stools in an eight hour period or more than three unformed stools in a 24 hour period if accompanied by other enteric symptoms). In that study, seven out of 18 subjects (39%) had diarrhea with or without other symptoms; and the development of symptoms was not correlated with the size of the dose (4, 10). The present analysis assumes that the volunteer data are generally applicable, but that certain segments of the non-AIDS population (e.g. children) may be more prone to symptomatic illness; therefore 0.5 was selected for pD, the probability of diarrheal illness following infection. For the AIDS population, a probability of 1.0 was selected.

p(moderate-severe illness | diarrhea), pM. Morbidity in the moderate-severe range is defined here as diarrheal illness that has resulted in severe impairment or moderate impairment which is not improving after one week. In the volunteer study, of the seven diarrheal subjects, the maximum duration of diarrhea was only four days, and none of the illnesses were characterized as severe (4, 15). Data from outbreaks must be treated with caution due to the possibilities of reinfection, multiple etiologies, etcetera. Nonetheless, community surveys performed during outbreaks have indicated a median diarrhea due to *Cryptosporidium* infection of about 3 days (9, 16). This analysis estimated that approximately 5-10% of infections will result in severe morbidity in the early acute phase, and that an additional 5-10% will meet the prolonged duration criteria. Therefore, for the non-AIDS population, a value of 0.15 was ascribed to pM. For persons with AIDS, *Cryptosporidium* infections tend not to be self-limiting when CD4 counts are below 180 (17) and the occurrence of chronic diarrhea increases as the CD4 count declines; a value of 0.95 was selected for pM in this population.

p(physician visit | moderate-severe illness), pV. The analysis thus far provides a subset of infections which might reasonably be expected to be associated with medical care. However, physician contact for diarrhea is likely to be influenced by factors reflecting illness severity, access to care, and other personal factors such as tendencies or biases towards seeking physician care. A consideration which may decrease the tendency to seek and receive in-person care even in those with moderate-severe *Cryptosporidium* infection are the frequent absences of systemic illness, fever, and abdominal pain (16). For the non-AIDS population, a value of 0.4 was selected for pV. Combining the estimates for the non-AIDS population gives an overall physician visit rate of 6% (given that diarrheal illness has occurred), which is similar to available estimates (16). For persons with AIDS, a probability of 0.9 was selected because they are generally under a regular and frequent schedule of medical care and the diarrheal symptoms are very likely to be prolonged and therefore present when the patient is seen by a physician.

p(ova & parasite exam | physician visit), pO. Ova and parasite (O&P) testing is recommended when warranted by clinical suspicion and/or when the illness is medically important (e.g. immunocompromised host or a case with severe volume depletion, bleeding, high fever, etc.), unresponsive, or prolonged (18). Decreasing the likelihood that an O&P will be ordered are the possibilities that a) other therapy and/or testing would take place first, with subsequent improvement; b) the absence of unusual circumstances (e.g. foreign travel) which might arouse clinical suspicion of parasitic infection, and c) questions regarding the cost effectiveness and value of stool testing. In addition, even if a physician orders an O&P test, ambulatory patients may not submit requested samples, or may do so in an inappropriate or untimely manner. For the non-AIDS group, it was estimated that between a third and a half of acute case-patients would have O&P exams ordered, providing an estimate of 0.4 for pO. Diarrheal disease in persons with HIV infection significantly affects the individual's health, prognosis, and management (19). In such persons, aggressive testing for an etiologic agent of chronic diarrhea has been described and recommended. For these reasons, a probability of 0.9 was selected for persons with AIDS.

p(*Cryptosporidium* test | ova & parasite exam), pC. The primary benefit of diagnosis from the clinical perspective is the exclusion of other causes, as no drug therapies for cryptosporidiosis have proven effective (20). Awareness among physicians of the symptoms of cryptosporidiosis is not universal, and

some physicians may mistakenly assume that it is a routine component of the O&P (20). For these reasons, it is likely that in the majority of O&P exams for patients who do not have AIDS, *Cryptosporidium* testing will not be requested. In the U.S., only 5% of laboratories performing O&P exams routinely screen for *Cryptosporidium* (20). This study ascribes a value of 0.1 to pC for the non-AIDS population. In persons with AIDS, we ascribe a probability of 0.95 for *Cryptosporidium* testing when O&P exams are ordered, because most physicians treating AIDS patients are aware of cryptosporidiosis as an opportunistic disease affecting the medical management of their patients.

p(case detection and reporting | *Cryptosporidium* test), pR. The likelihood that a cryptosporidiosis case will be diagnosed and reported to the appropriate health authority subsequent to a stool exam for *Cryptosporidium* depends on the sensitivity of the testing and the effectiveness of the reporting system. The overall sensitivity of testing will be affected by the number of exams performed, the intermittence, duration, and intensity of oocyst shedding, and the laboratory's diagnostic sensitivity and proficiency. The volunteer study demonstrated that for subjects with diarrheal illness, the median percentage of positive stools during illness was about 60% (15). Most clinical laboratories use a screening method which is less sensitive (20). This could be offset by instances in which multiple samples are submitted. With respect to the reporting system, an active surveillance system for cryptosporidiosis which is 100% effective at soliciting case reports was assumed. Therefore, a value of 0.6 was assigned to pR for the non-AIDS populations. For persons with AIDS, there is a greater likelihood that oocyst shedding will be heavy and persistent; nearly 100% of AIDS-related *Cryptosporidium* infections can be detected with two samples (21). Therefore, pR was assigned a value of 0.95 in this population.

RESULTS AND DISCUSSION

Table 1 summarizes the central tendency values selected for the conditional probabilities. In contrast to persons with AIDS, in whom most infections (nearly 70%) were predicted to result in case reports, fewer than one in a thousand infections in the non-AIDS population are predicted to be reported. Rates of infection and other outcomes per 100,000 population are shown in Table 2. In the non-AIDS population, the average annual risks of tapwater-related *Cryptosporidium* infection were estimated as approximately 0.1 or 1% when the concentration of infective *C. parvum* oocysts was either one per 1,000 or 100 liters, respectively. Risks of infection are estimated to be about twice as high in persons with AIDS. In the non-AIDS population, the effects of the spectrum of outcomes and low likelihood of specific testing for *Cryptosporidium* infection are striking. Even at the high range estimate (one oocyst per hundred liters), with approximately 1,000 infections per 100,000 persons, only about 30 illnesses would be predicted to result in physician care, with only one reported case of cryptosporidiosis. In persons with AIDS, an annual incidence of detected cryptosporidiosis of 0.1-1.4% is predicted. Table 3 demonstrates the effect of persons with AIDS on community rates. Rates per 100,000 persons were calculated with the data derived in Table 2, using community prevalences of AIDS ranging from 0.1-0.4%. (This range is consistent with many large U.S. metropolitan areas; for example, New York City is at the high end of this range). The tapwater-related rates range from approximately 2 to 7 cases per 100,000 at the high concentration. The proportion of cases detected in persons with AIDS accounts for 66-89% of the estimated rates.

TABLE 1: Summary of conditional probabilities used to relate *Cryptosporidium* infection to case detection and reporting

	population	
conditional probability	non-AIDS	AIDS
pD, p(diarrheal illness | infection)	0.50	1.00
pM, p(moderate-severe illness | D)	0.15	0.95
pV, p(physician visit | M)	0.40	0.90
pO, p(ova & parasite exam | V)	0.40	0.90
pC, p(*Cryptosporidium* test | O)	0.10	0.95
pR, p(case detection & reporting | C)	0.60	0.95
infections leading to case reports	0.07%	69%

TABLE 3: Predicted rates and proportions of reported cryptosporidiosis related to tapwater with assumed *C. parvum* concentrations, according to the community prevalence of AIDS

	predicted rate* of reported cases, per 100,000		
	C. parvum concentration (oocysts/L)		proportion due to AIDS-related cases
prevalence of AIDS	0.001	0.01	
0.1%	0.2	2.2	66%
0.2%	0.4	3.6	79%
0.3%	0.5	5.1	85%
0.4%	0.7	6.5	89%

* Calculated with rates derived in Table 2

TABLE 2: Estimated annual risks of infection and numbers of related outcomes per 100,000 persons in AIDS and non-AIDS populations from consumption of tap water with assumed concentrations of *C. parvum*

Parameter	Population				Units
	non-AIDS		AIDS		
A. assumed *C. parvum* concentration	0.001	0.01	0.001	0.01	[oocysts/L]
B. average tapwater intake	247	247	165	165	[L/person-year]
C. average annual exposure (*A x B*)	0.247	2.47	0.165	1.65	[oocysts/person-yr]
D. infectivity	0.0042	0.0042	0.0126	0.0126	[infection/oocyst]
E. average annual risk of infection (*C x D*)	0.0010	0.0104	0.0021	0.0207	[infections/person-yr]
F. population	100,000	100,000	100,000	100,000	[persons]
G. annual infections (*E x F*)	104	1,035	207	2,075	[infections/yr]
H. probability of diarrheal illness, pD	0.50	0.50	1.00	1.00	[diarhheal illness/infection]
I. diarrheal illnesses (*G x H*)	52	518	207	2,075	[diarrheal illnesses/yr]
J. probability of mod-severe illness	0.15	0.15	0.95	0.95	[mod-severe illness/illness]
K. moderate-severe illnesses (*I x J*)	8	78	197	1,971	[mod-severe illnesses/yr]
L. probability of physician visit, pM x pV	0.40	0.40	0.90	0.90	[physician-case/illness]
M. cases seen by physician (*L x M*)	3	31	177	1,774	[physician-cases/yr]
N. probability of Ova & parasite exam, pO	0.40	0.40	0.90	0.90	[O&P exam/physician-case]
O. probability of *Cryptosporidium* test, pC	0.10	0.10	0.95	0.95	[*C.* test/O&P exam]
P. probability of detection/reporting, pR	0.60	0.60	0.95	0.95	[case report/*C.* test]
Q. estimated reported cases (*M x N x O x P*)	0	1	140	1,400	[case reports/100,000 person-yr]

It appears that the risk assessment predictions are reconcilable with endemic levels of cryptosporidiosis. The limited data available from cryptosporidiosis surveillance programs indicate overall rates in the range of 1-10 per 100,000 persons/year (3, 22). For example, in 1995, New York City's active surveillance demonstrated a rate of approximately six cases per 100,000, with about 85% of cases having occurred in persons with HIV infection (23).

Two factors prevent an evaluation of whether the risk assessment approach can truly be considered validated. First, the fraction of endemic cases (in New York City and elsewhere) which are actually due to tapwater consumption is unknown. Second, the actual occurrence of infective *C. parvum* in tapwater has not been accurately characterized due to limitations inherent in the methodologies. This analysis shows that the preponderance of detected cases among persons with AIDS is not inconsistent with a common exposure route such as tapwater. Risk assessment cannot be used to prove that any endemic *Cryptosporidium* infections are waterborne in origin. However, the epidemiologic findings thus far have not ruled out a role for waterborne transmission in endemic cryptosporidiosis. Tapwater could represent a tenth or a half of the detected cases of disease and yet be difficult to demonstrate in an epidemiologic investigation (6). There is a clear need for improved epidemiologic investigations of the role of water relative to other exposures, especially for persons with AIDS (8).

A comparison with giardiasis reveals a seeming incongruity. Because disinfection practices are believed to be highly effective, it is assumed that the risk of acquiring *Giardia* infection from tapwater is eliminated in most systems with a high quality source, such as New York City's. However, giardiasis remains common; in New York City there are about four to five times as many giardiasis cases reported as there are cryptosporidiosis cases (22). Since there may be about ten times as much stool testing for *Giardia*, the endemic levels may be similar. Although there is no reason to expect that the two pathogens should follow identical patterns of transmission, the *Giardia* example suggests that tapwater is not a necessary exposure route for a significant level of endemic disease to be present in the general population.

Although the uncertainties inherent in the risk assessment are substantial, it is likely that the uncertainty contributed by the concentration parameter will overshadow the uncertainty in the other parameters in our analysis. This was dealt with by presenting results for a range of concentrations, consistent with *Cryptosporidium* monitoring performed to date throughout the U.S. Future refinements in monitoring may confirm that this range is appropriate, or possibly that actual concentrations are lower. A quantitative estimate of the spectrum of outcomes for *Cryptosporidium* infection, similar to the approach developed in this analysis, may provide data that are suitable for use in cost-benefit analysis, representing a refinement of previous approaches which assigned costs as if every infection was equivalent to a detected illness (24). However, evaluations of treatment needs will have to consider that regardless of the level of water quality targeted or achieved, risks to persons with AIDS or other severe immune disorders will be difficult to eliminate.

ACKNOWLEDGMENTS

This work was supported by N.I.H. grant A126497 in the International Collaborative Infectious Disease Research Program to S.M.L.B., the Center for Environmental Research and Conservation (Columbia University), and a Graduate Fellowship from the U.S. Environmental Protection Agency (EPA) to J.F.P.

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Appendix B:

Evaluation of the Uncertainty Associated with Estimating the Concentration of *Cryptosporidium* in Tap Water

An evaluation of the uncertainty associated with a central tendency estimate of the true concentration of infective *C. parvum* derived from monitoring data can be made as follows. Consider the following information, typical of data from published reports for finished surface water that is ready for distribution [Rose, 1991 #106; LeChevallier, 1991 #120; LeChevallier, 1995 #164]:

- 15% of samples had positive findings for *Cryptosporidium*
- the mean concentration for positive samples was 0.033 oocysts per liter
- typical analyzed volumes were approx. 50 liters (i.e., 0.02 detection limit)

Assuming the analyzed volume of each sample was similar, a nominal mean concentration can be estimated from the pooled results:

$$C_{NOMINAL} \cong 0.15 \times 0.033 \cong 0.005 \text{ oocysts/liter}$$

The true concentration may be approximated from $C_{NOMINAL}$ by taking into account the recovery efficiency of the assay, the fraction of *C. parvum* which are viable, and the fraction of detected organisms which are actually pathogenic (*C. parvum*):

$$C_{TRUE} \cong C_{NOMINAL} \times (1/RECOVERY) \times VIABILITY \times PATHSPP$$

Fairly conservative estimates of these modifying parameters, with lognormal distributions, can be made by considering available information [Rose, 1991 #106; LeChevallier, 1991 #120; LeChevallier, 1995 #164; Centers for Disease Control and Prevention, 1995 #209]:

parameter	central tendency	95% CI range	dispersion factor, k
RECOVERY	0.10	0.02-0.50	5
VIABILITY	0.10	0.02-0.60	6
PATHSPP	0.50	0.25-1.00	2

where the dispersion factor describes the 95% confidence interval (see footnotes to Table 2-1 for details). C_{TRUE} can then be calculated as 0.005 x (1/0.08) x 0.10 x 0.50 ≅ 0.003 oocyst/liter, with a 95% CI 0.0002-0.04, representing a 150-fold range. Note that the uncertainty in this value was estimated by calculating the overall dispersion factor:

$$k_T = \exp\{\sqrt{(\ln^2 5 + \ln^2 6 + \ln^2 2)}\} \cong 12.3$$

$$95\%CI = \{ (C_{TRUE} / k_T) \text{ to } (C_{TRUE} \times k_T) \}$$

Appendix C:

Permit from the New York City Department of Environmental Protection to Conduct Sampling in the Vicinity of the New Croton Reservoir

THE CITY OF NEW YORK DEPARTMENT OF ENVIRONMENTAL PROTECTION
JOEL A. MIELE, SR., P.E. Commissioner

WILLIAM N. STASIUK, P.E.,Ph.D.
Deputy Commissioner

PHONE (914) 742-2070
FAX (914) 742-2040

Bureau of Water Supply,
Quality and Protection
Office of Water Supply Lands (OWSL)

August 26, 1997

Sylvia M. Le Blancq, Ph.D.
Assistant Professor of Public Health
Columbia School of Public Health
60 Haven Avenue, B-1
New York, New York 10032-4206

Re: Cryptosporidium Study

Dear Ms. LeBlancq:

This is in response to your letter dated August 13, 1997 to Marilyn Shanahan, Manager, Office of Water Supply Lands requesting a one (1) year extension of the offer dated September 4, 1996 (copy enclosed) for accessing various areas of City-owned water supply lands adjacent to the New Croton Reservoir in the Towns of New Castle and Bedford, Westchester County for the purpose of conducting a cryptosporidium study which includes examining the potential role of white-tailed deer as carriers of cryptosporidium.

This Department has no objections to extending the aforementioned offer until September 9, 1998.

If you have any questions concerning this matter please contact Marilyn Shanahan at 9140742-2070.

Very truly yours,

William Stasiuk, P.E. , Ph.D.

Encl.

The City of New York

DEPARTMENT OF ENVIRONMENTAL PROTECTION

EXECUTIVE OFFICES
59-17 JUNCTION BLVD., 19TH FLOOR, CORONA, NEW YORK 11368-5107

JOEL A. MIELE, SR., P.E., COMMISSIONER

(718) 595-6565 FAX: (718) 595-3525

September 4, 1996

Sylvia M. Le Blancq, Ph.D.
Columbia University
Division of Environmental Health Sciences
630 West 18th Street, VC15-220
New York, New York 10032

Re: Cryptosporidium Study

Dear Dr. Le Blancq:

This is in response to your letter dated August 15, 1996 to Marilyn Shanahan, Manager, Office of Water Supply Lands requesting permission to access various areas of City-owned water supply lands adjacent to the New Croton Reservoir in the Towns of New Castle and Bedford, Westchester County for the purpose of conducting a cryptosporidium study which includes examining the potential role of white-tailed deer as carriers of cryptosporidium.

There is no objection to granting such permission subject to the following conditions:

1. This permission is limited to you and two (2) assistants for a one (1) year period only and must commence and conclude between September 9, 1996 and September 9, 1997.

2. The collection and study of samples must be coordinated with this Department's Division of Water Quality Control's Pathogen Wildlife Study. David Stern, telephone number 914-773-4430 must be contacted prior to the commencement of this study.

3. This Department's District Engineer, Carl Picha, P.E., telephone number 914-232-5171 and Michael Collins, Director, DEP Police, telephone number 914-742-2011 must be notified at least 48 hours before entering upon City property.

4. All civil, state, county and town laws and regulations applicable to this study shall be complied with.

Columbia University
New York, New York 10032

Re: Cryptosporidium Study
September 4, 1996

5. You and your assistants must agree to save the City of New York harmless from any and all damages or injury to persons or property arising from the use of City land or structures.

6. You and your assistants shall assume full responsibility for any injury including death, or damages which may occur to you by reason of being on or about City property by signing the enclosed **RELEASES** before entering City property. Entry onto City property may not occur until such time as the required **RELEASES** have been submitted to and accepted by this Department's Office of Water Supply Lands. A copy of the signed **RELEASE** and proper identification must be carried with you and your assistants during the occupation of City property for presentation upon request.

7. Copies of any reports/results of the study are to be furnished to this Department's Office of Water Supply Lands, 465 Columbus Avenue, Suite 350, Valhalla, New York 10595-1336.

Please submit the completed **RELEASES** as indicated in special condition number five (5) and this authorization letter signed and notarized below to Marilyn Shanahan, Manager, Department of Environmental Protection, Bureau of Water Supply, Quality and Protection, Office of Water Supply Lands, 465 Columbus Ave., Suite 350, Valhalla, New York 10595-1336 before entering upon City property.

Very truly yours,

William N Stasiuk
by VH

William N. Stasiuk, P.E., Ph.D.
Deputy Commissioner

Encls.

Terms and conditions of this letter accepted by:

NAME SYLVIA M. LEBLANC, PHD SIGNATURE Sylvia LeBlanc

TITLE ASSISTANT PROFESSOR NOTARY PUBLIC

DATE SEPTEMBER 11, 1996

MOTTY GOLDSTEIN
Notary Public, State of New York
No. 24-4954078
Qualified in Kings County
Commission Expires July 31, 199

Columbia University in the City of New York | *New York, N.Y. 10032*

SCHOOL OF PUBLIC HEALTH
Division of Environmental Sciences

60 Haven Avenue, B-1
Cable Address:
COLUHEALTH New York
Telephone: (212) 305-3464
FAX: (212) 305-4012

Division of Environmental Health Sciences
630 West 168th Street, VC15-220
tel 212 305 2654
fax 212 305 4496
e-mail SML43@columbia.edu

Ms. M. Schanahan
Manager, Office of Water Supply and Lands
465 Columbus Avenue, Suite 350
Valhalla
NY 10595

August 15, 1996

Dear Ms. Schanahan,

I am writing to request permission for access to New York City property along the New Croton Reservoir in New Castle and Bedford.

We are studying *Cryptosporidium* in New York and part of the project is to examine the potential role of white-tailed deer as carriers of *Cryptosporidium*. We would like access to New York City property so that we can survey a deer population in the Croton watershed. Such a survey would entail the following:

1. collection of deer scat at two to three week intervals over a period of one year along the edge of the New Croton Reservoir (in New Castle and Bedford). This will only involve the collection of deer droppings.
2. The scat will then be tested for the presence of oocysts in my laboratory at Columbia University.

We will forward a copy of the results to you at the end of the study.

I look forward to hearing from you.
Thank you very much,

Sincerely,

Sylvia Le Blancq

Sylvia M. Le Blancq, Ph.D.
Assistant Professor of Public Health

Appendix D:

Institutional Review Board Letter
re: Analysis of Human Isolates (waiver)

COLUMBIA UNIVERSITY
COLLEGE OF PHYSICIANS & SURGEONS

COLUMBIA-PRESBYTERIAN MEDICAL CENTER INSTITUTIONAL REVIEW BOARD
CPMC IRB

DATE: January 14, 1999

TO: Sylvie Le Blancq, M.D.
School of Public Health
60 Haven Av, B-1

FROM: Rosemarie Price, Associate Administrator RPrice

RE: IRB APPROVAL OF RESEARCH PROTOCOL

The research study involving human subjects described in the protocol you recently submitted for IRB review (IRB # and title listed below) was ratified at the IRB meeting held Wednesday, January 13, 1999.

This meeting date is the official date of approval for your protocol and is the date to be used on all certification forms (including HHS form 596) or letters forwarded to funding agencies.

IRB #X0746, THE EPIDEMIOLOGY OF CRYPTOSPORIDIUM INFECTION: MOLECULAR BIOLOGY AND MATHEMATICAL MODELING APPROACHES",

RP/jo

630 West 168th Street P&S 3-460 New York, NY 10032 212-305-5883 Fax 212-305-1316

COLUMBIA UNIVERSITY
COLLEGE OF PHYSICIANS & SURGEONS

COLUMBIA-PRESBYTERIAN MEDICAL CENTER INSTITUTIONAL REVIEW BOARD
CPMC IRB

January 11, 1999

Dr.Sylvie LeBlanq
Division of Environmental Sciences
School of Public Health
60Haven Avenue, B-1

RE: IRB #0746, "THE EPIDEMIOLOGY OF *CRYPTOSPORIDIUM* INFECTION: MOLECULAR BIOLOGY AND MATHEMATICAL MODELING APPROACHES"

Dear Dr. LeBlanq:

Thank you for your letter of January 6, 1999. Since this study involves only the use of existing specimens without patient identifiers, it is exempt from IRB review requirements. We are enclosing a signed copy of the approved exempt study with this letter.

The study will be presented at the next IRB meeting for confirmation of its exempt status; however, you may proceed with the study as of today's date.

Please keep us informed should the scope of the research change so as to require review.

Sincerely,

Donald S. Kornfeld, M.D., Chairman
CPMC Institutional Review Board

DSK:jlv

cc: John Colombotos, Ph.D.

630 West 168th Street P&S 3-460 New York, NY 10032 212-305-5883 Fax 212-305-1316

Columbia School *of* Public Health

CSPH

Division of Environmental
Health Sciences

Columbia University
Health Sciences Division
Institutional Review Board
630 West 168th Street
P&S Building 3-460
Attn.: Dr. Donald Kornfield, MD, Chairman

Sept. 28, 1998

Re: *Cryptosporidium* study

Dear Dr. Kornfield,

We are writing to request an exemption from IRB review for the protocol described herein. The project involved represents a doctoral dissertation entitled "The Epidemiology of *Cryptosporidium* Infection: Molecular Biology and Mathematical Modeling Approaches."

One of the primary aims of the project concerns the molecular typing of the enteric parasite *Cryptosporidium*. In order to accomplish this, we need to obtain stool from patients who have received a diagnosis of *Cryptosporidium* infection. These specimens will already have been collected and analyzed for clinical purposes, and would be made available to us. To assure confidentiality, we have arranged that the sample will be identified by a number, only. After the necessary material has been recovered, the unused portion of each sample will be discarded in accordance with University policies. Therefore, we request that this protocol be granted an exemption by the IRB. Please do not hesitate to contact us if you require further information.

Sincerely,

Joseph F. Perz
doctoral candidate
Division of Environmental Health Sciences
School of Public Health

APPROVED: _____ 10/29/98
Donald S. Kornfeld, M.D., Chairman
CPMC Institutional Review Board

Dr. Sylvie M. Le Blancq
Assistant Professor and Thesis Advisor
Division of Environmental Health Sciences
School of Public Health

RECEIVED OCT 21 1998

60 Haven Avenue, B-1
New York, NY 10032-4206

PHONE 212-305-3464
FAX 212-305-4012

Appendix E:

Protocol for the Isolation and Purification of DNA from Stool Samples Obtained from Human Patients

Protocol for the isolation and purification of DNA from stool samples obtained from human patients

Note: Processing of samples must be done in a designated area separate from the PCR set-up and post-PCR zones (ideally, these should be in different rooms). Protective clothing must be worn at all times and universal precautions must be strictly observed. Work pertaining to manipulation of specimens in steps 1-7 or organic extractions must be performed behind a protective barrier in the appropriate hood.

A. Wash/concentrate

1. Decant specimen into a sterile 50 ml tube, make up volume to 50 ml with cold PBS, mix by shaking, centrifuge 5 min @ 500 g and decant supernatant.
2. Repeat wash with 100% ethanol. (This step may be omitted if specimen was not provided in preservative)
3. Add approximately 10 ml cold PBS and mix.
4. Pour slurry through funnel-screening device into 50 ml tube, washing with additional PBS to 50 ml; shake, centrifuge 5 min @ 500 g and decant supernatant. (Screening may be omitted if only a small amount of sediment is present.)
5. Resuspend sediment with the use of a 2 ml transfer pipette and additional PBS as needed; transfer up to 500 mg to 2 ml tube(s) with screw top/o-ring and centrifuge 1 min @ 16,000 g (max speed).
6. Remove supernatant and record net weight of sediment.

B. Lysis

7. Suspend sediment with 1.25 ml DNAzol; fill tube w/ 2-3 grams 0.1 mm zirconium-silica beads.
8. Homogenize sample for 5 min at 4200 rpm using a Mini-Beadbeater; hold on ice.
9. Incubate in water bath for 10 min @ 90°C and then hold samples on ice.
10. Recover lysate as follows. Centrifuge 2 min @ 1000g and transfer 0.75 ml supernatant to new tube. Add 0.75 ml chloroform to the original sample tube, shake 20 sec, hold 2 min, centrifuge 5 min @ 1000g. Combine available supernatant to tube containing previously recovered lysate.

C. Purification and Precipitation

11. Transfer 1ml lysate to a 2 ml tube pre-filled with 1 ml Phenol:Chloroform:Isoamyl Alcohol (25:24:1).

(continued below)

Protocol for the isolation and purification of DNA from stool samples obtained from human patients (continued)

12. Shake vigorously 20 sec, hold at room temp 3 min, centrifuge 7 min @ 16,000 g
13. Transfer lysate to 2 ml tubes pre-filled with 0.9 ml Chloroform:Isoamyl Alcohol (24:1).
14. Shake vigorously 20 sec, hold at room temp 3 min, centrifuge 7 min @ 16,000 g
15. Transfer 0.8 ml lysate to 1.5 ml tube pre-filled with 5 µl polyacryl carrier plus 0.4 ml (room temperature) 100% ethanol.
16. Invert 8-10 times, hold at room temp 3 min, centrifuge 5 min @ 5,000 g.

D. Washing and Elution

17. Carefully remove supernatant by decanting; wash w/ 1 ml 95% ethanol, raising pellet by gentle vortexing or vigorously inverting tube.
18. Hold at least 5 min at room temperature, centrifuge 2 min @ 5,000 g.
19. Wash w/ 1 ml 95% ethanol as above.
20. Carefully remove ethanol by pipetting, allow pellet to air dry for approximately 10 min.
21. Add 100 µl sterile H_20, incubate at 65°C for 5 min, solubilize w/ gentle vortexing as needed, and hold on ice.
22. Centrifuge 10 min @ 16,000 g to remove insoluble material.
23. Create 0.1X dilution with sterile H_2O.

Materials

15 ml tube (Falcon)
50 ml tube (Falcon)
applicator sticks (Fisher)
funnel-screening device (Meridien Contrate)
transfer pipette (Fisher)
2 ml tube (screw top, O-ring) (Biospec)
DNAzol (MRC)
0.1 mm zirconium-silica beads (Biospec)
Mini-Beadbeater (Biospec)
26G1/2 needle
Phenol:Chloroform:Isoamyl Alcohol, 25:24:1 (Gibco Life)
polyacryl carrier (MRC)
sterile (molecular biology grade) H_2O (5prime3prime)

Appendix F:

Results of BLAST Analysis for the SSU Product Sequences from Selected Wildlife Samples

NCBI **BLAST Search Results** Entrez ?

Commencing search, please wait for results.

BLASTN 2.0.8 [Jan-05-1999]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query= 569 392nt
(392 letters)

Searching.....done

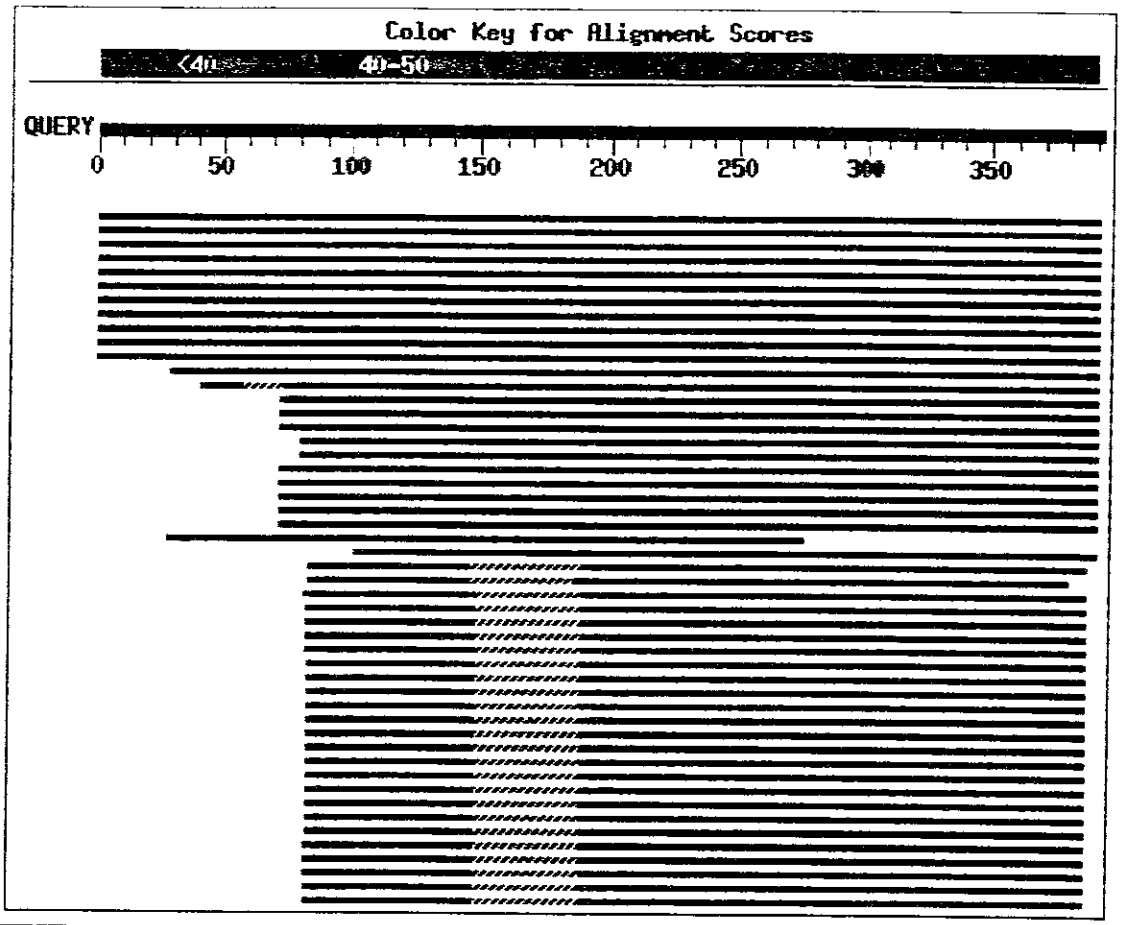
If you have any problems or questions with the results of this search please refer to the **BLAST FAQs**

Distribution of 76 Blast Hits on the Query Sequence

Mouse-over to show defline and scores. Click to show alignments

569S (MINUS PRIMERS, REPEAT MASKER OFF)

392 N.T.
TTAATAATTTATATATAATATTTTATTAATATTTATATATTATTAACATAATTCATATT
ACTATATTTTATAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATTAGCCTTGAATACTCCAGC
ATGGAATAATATAAAAGATTTTTATCTTTTTTATTGGTTTTAAGATAAAAATAATGATTAATAGGGACAGTTGGGGGCAT
TTGTATTTAACAGTCAGAGGTGAAATTCTTAGATTTGTTTAAGACAAACTAGTGCGAAAGCATTTGCCAAGGATGTTTTC
ATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCAACTAGAG
ATTGGAGGTTGTT



Sequences producing significant alignments:	Score (bits)	E Value
gb|U11440|CWRASRRN Cryptosporidium wrairi small subunit ribosom...	684	0.0
gb|AF087576|AF087576 Cryptosporidium parvum from patient 53 18S...	676	0.0
gb|AF040725|AF040725 Cryptosporidium parvum external transcribe...	676	0.0
gb|AF015772|CPRRNAA1 Cryptosporidium parvum small subunit ribos...	676	0.0
gb|L16996|CYDRG18SA Cryptosporidium parvum 18S ribosomal RNA ge...	676	0.0
gb|AF093492|AF093492 Cryptosporidium parvum strain Human C. par...	670	0.0
gb|AF087575|AF087575 Cryptosporidium parvum from patient 124 18...	662	0.0
gb|L16997|CYDRG18SB Cryptosporidium parvum 18S ribosomal RNA ge...	662	0.0
gb|AF093491|AF093491 Cryptosporidium parvum strain Human C. par...	662	0.0
gb|AF087574|AF087574 Cryptosporidium parvum from patient 115 18...	624	e-177
~~gb|L25642|CYDRG18S Cryptosporidium parvum 18S ribosomal RNA (18...~~	~~607~~	~~e-172~~ ①
gb|L19068|CYDRGEA Cryptosporidium baileyi 18S ribosomal RNA gen...	577	e-163
gb|AF087577|AF087577 Cryptosporidium ~~parvum~~ from patient 84 18S...	567	e-160 ②
gb|AF093499|AF093499 Cryptosporidium serpentis strain Snake C. ...	476	e-132
gb|AF093501|AF093501 Cryptosporidium serpentis strain Savannah ...	468	e-130
gb|L19069|CYDRGEB Cryptosporidium muris 18S ribosomal RNA gene.	468	e-130
gb|AF093496|AF093496 Cryptosporidium muris strain Calf C. muris...	468	e-130
gb|AF093500|AF093500 Cryptosporidium serpentis strain Savannah ...	468	e-130

note: ① invalid
② felis

emb|X64341 CPL18SR931	C.parvum ribosomal RNA gene for 18S rRNA (...	460	e-128
gb|AF093497 AF093497	Cryptosporidium muris strain Bactrin Camel...	460	e-128
emb|X64342 CML18SR221	C.muris ribosomal RNA gene for 18S rRNA (...	456	e-126
emb|X64340 CPL18SR911	C.parvum ribosomal RNA gene for 18S rRNA (...	454	e-126
emb|X64343 CML18SR206	C.muris ribosomal RNA gene for 18S rRNA (p...	436	e-120
gb|S76662|S76662	18S rRNA [Cryptosporidium parvum, calf isolate...	422	e-116
gb|S71380|S71380	18S rRNA [Cryptosporidium parvum, Genomic, 556...	412	e-113
gb|L19080|CXEFF18S	Cytauxzoon felis 18S ribosomal RNA.	337	9e-91
gb|AF006471 AF006471	Sarcocystis hominis 18S ribosomal RNA gene...	337	9e-91
dbj|AB012189|AB012189	Theileria sp. gene for small subunit rRNA...	329	2e-88
gb|U97054|TSUF7054	Theileria sp. 18S ribosomal RNA type F gene,...	329	2e-88
dbj|AB012194|AB012194	Theileria sp. gene for small subunit ribo...	329	2e-88
dbj|AB012199|AB012199	Theileria sp. gene for small subunit ribo...	329	2e-88
gb|AF097993|AF097993	Theileria velifera 18S ribosomal RNA gene,...	329	2e-88
gb|U97051|TSUF7051	Theileria sp. 18S ribosomal RNA type C gene,...	329	2e-88
dbj|AB000271|AB000271	Theileria sergenti gene for small subunit...	329	2e-88
dbj|AB000273|AB000273	Theileria sp. gene for small subunit ribo...	329	2e-88
gb|U97053|TSUF7053	Theileria sp. 18S ribosomal RNA type E gene,...	329	2e-88
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dbj|AB000274|AB000274	Theileria sp. gene for small subunit ribo...	329	2e-88
dbj|AB000270|AB000270	Theileria sp. gene for small subunit ribo...	329	2e-88
dbj|AB000272|AB000272	Theileria buffeli gene for small subunit ...	329	2e-88
gb|U97048|TSUF7048	Theileria sp. 18S ribosomal RNA type B gene,...	329	2e-88
gb|U97052|TSUF7052	Theileria sp. 18S ribosomal RNA type D gene,...	329	2e-88
emb|Z15106 TEL18SRRN	T.buffeli gene encoding 18S ribosomal RNA >...	329	2e-88
gb|AF036336|AF036336	Theileria sp. China 18S ribosomal RNA gene...	329	2e-88
gb|M64243|THEPR16S	Theileria annulata 16S-like ribosomal RNA (1...	325	4e-87
dbj|AB012196|AB012196	Theileria sp. gene for small subunit ribo...	321	6e-86
gb|L02366|THEI8SRRNA	Theileria parva 18S ribosomal RNA (18S rRN...	321	6e-86
gb|AF013418|AF013418	Theileria parva small subunit ribosomal RN...	321	6e-86
gb|L19082|THEPR18SA	Theileria taurotragi 18S ribosomal RNA.	321	6e-86

gb|U11440 CWPASRRN Cryptosporidium wrairi small subunit ribosomal RNA gene.
Length = 1746

Score = 684 bits (345), Expect = 0.0
Identities = 382/393 (97%), Gaps = 1/393 (0%)
Strand = Plus / Plus

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Query: 120  ttagccttgaatactccagcatggaataatataaaagattttatctttttattggttc 179
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Sbjct: 742  ttagccttgaatactccagcatggaataatattaaagattttatctttcttattggttc 801
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note: ③ muris

```
Query: 180  taagataaaaataatgattaatagggacagttgggggcatttgtatttaacagtcagagg 239
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Sbjct: 802  taagataagaataatgattaatagggacagttgggggcatttgtatttaacagtcagagg 861

Query: 240  tgaaattcttagatttgttaaagacaaactagtgcgaaagcatttgccaaggatgttttc 299
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gb|AF087576|AF087576 Cryptosporidium parvum from patient 53 18S ribosomal RNA gen
partial sequence
Length = 435

Score = 676 bits (341), Expect = 0.0
Identities = 381/393 (96%), Gaps = 1/393 (0%)
Strand = Plus / Plus

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Query: 60   actatattttatagtatatgaaattttactttgagaaaattagagtgcttaaagcaggca 119
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Query: 300  attaatcaagaacgaaagttaggggatcgaagacgatcagataccgtcgtagtcttaacc 359
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```

586/4275

NCBI **BLAST Search Results** Entrez ?

Commencing search, please wait for results.

586S & 4247S (MINUS PRIMERS, REPEAT MASKER OFF)

391 N.T.

TTAATAATTTATATATAATATTTTATGATATTTATATAATATTAACATAATTCATTATTA
CTATTATTATTAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCTTTTGCCTTGAATACTCCAGCA
TGGAATAATATAAAAGATTTTTATCTTTTTTATTGGTTCTAAGATAAAAATAATCATTAATAGGGACAGTTGGGGGCATT
TGTATTTAACAGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTAGTGCGAAGCATTTGCCAAGGATGTTTTCA
TTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCAACTAGAGA
TTGGAGGTTGTT

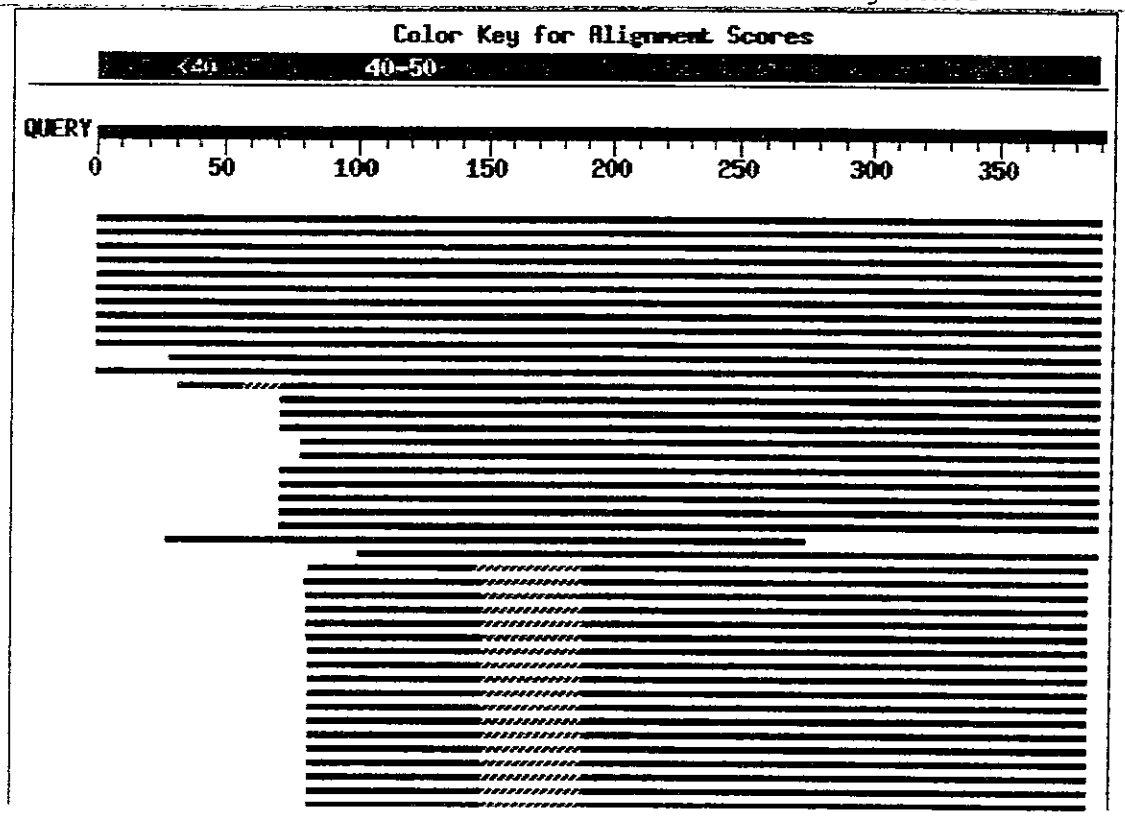
Query= 586|4247|ssu
(391 letters)

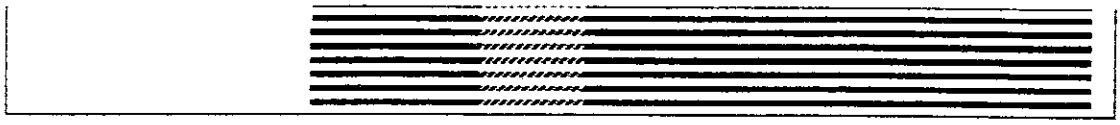
Searching.....done

If you have any problems or questions with the results of this search please refer to the **BLAST FAQs**

Distribution of 76 Blast Hits on the Query Sequence

Mouse-over to show defline and scores. Click to show alignments





Sequences producing significant alignments:	Score (bits)	E Value
gi|U11440|CWRASRRN Cryptosporidium wrairi small subunit ribosom...	684	0.0
gi|AF093492|AF093492 Cryptosporidium parvum strain Human C. par...	670	0.0
gi|L16997|CYDRG18SB Cryptosporidium parvum 18S ribosomal RNA ge...	662	0.0
gi|AF093491|AF093491 Cryptosporidium parvum strain Human C. par...	662	0.0
gi|L16996|CYDRG18SA Cryptosporidium parvum 18S ribosomal RNA ge...	662	0.0
gi|AF087576|AF087576 Cryptosporidium parvum from patient 53 18S...	662	0.0
gi|AF087575|AF087575 Cryptosporidium parvum from patient 124 18...	662	0.0
gi|AF015772|CPRRNAA1 Cryptosporidium parvum small subunit ribos...	662	0.0
gi|AF040725|AF040725 Cryptosporidium parvum external transcribe...	662	0.0
gi|AF087574|AF087574 Cryptosporidium parvum from patient 119 18...	642	0.0
gi|L19068|CYDRGEA Cryptosporidium baileyi 18S ribosomal RNA gen...	595	e-168
~~gi|L25642|CYDRG18S Cryptosporidium parvum 18S ribosomal RNA (18...~~	~~593~~	~~e-167~~ ①
gi|AF087577|AF087577 Cryptosporidium parvum from patient 84 18S...	583	e-165 ②
gi|AF093499|AF093499 Cryptosporidium serpentis strain Snake C. ...	476	e-132
gi|AF093501|AF093501 Cryptosporidium serpentis strain Savannah ...	468	e-130
gi|L19069|CYDRGEB Cryptosporidium muris 18S ribosomal RNA gene.	468	e-130
gi|AF093496|AF093496 Cryptosporidium muris strain Calf C. muris...	468	e-130
gi|AF093500|AF093500 Cryptosporidium serpentis strain Savannah ...	468	e-130
emb|X64341|CP18SR931 C.parvum ribosomal RNA gene for 18S rRNA (...	460	e-128 ③
gi|AF093497|AF093497 Cryptosporidium muris strain Bactrin Camel...	460	e-128
emb|X64342|CM18SR221 C.muris ribosomal RNA gene for 18S rRNA (...	456	e-126
emb|X64340|CP18SR911 C.parvum ribosomal RNA gene for 18S rRNA (...	454	e-126 ③
emb|X64343|CM18SR206 C.muris ribosomal RNA gene for 18S rRNA (p...	436	e-120
gi|S76662|S76662 18S rRNA [Cryptosporidium parvum, calf isolate...	416	e-114
gi|S71380|S71380 18S rRNA [Cryptosporidium parvum, Genomic, 556...	412	e-113
gi|L19080|CXZRR18S Cytauxzoon felis 18S ribosomal RNA.	337	9e-91
gi|U97054|TSU97054 Theileria sp. 18S ribosomal RNA type F gene,...	329	2e-88
dbj|AB012194|AB012194 Theileria sp. gene for small subunit ribo...	329	2e-88
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dbj|AB012189|AB012189 Theileria sp. gene for small subunit rRNA...	329	2e-88
gi|AF097993|AF097993 Theileria velifera 18S ribosomal RNA gene,...	329	2e-88
gi|U97051|TSU97051 Theileria sp. 18S ribosomal RNA type C gene,...	329	2e-88
dbj|AB000271|AB000271 Theileria sergenti gene for small subunit...	329	2e-88
dbj|AB000273|AB000273 Theileria sp. gene for small subunit ribo...	329	2e-88
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dbj|AB012202|AB012202 Theileria sp. gene for small subunit ribo...	329	2e-88
dbj|AB000274|AB000274 Theileria sp. gene for small subunit ribo...	329	2e-88
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dbj|AB000272|AB000272 Theileria buffeli gene for small subunit ...	329	2e-88
gi|U97052|TSU97052 Theileria sp. 18S ribosomal RNA type D gene,...	329	2e-88
gi|U97048|TSU97048 Theileria sp. 18S ribosomal RNA type B gene,...	329	2e-88
emb|Z15106|TB18SRRN T.buffeli gene encoding 18S ribosomal RNA >...	329	2e-88
gi|AF036336|AF036336 Theileria sp. China 18S ribosomal RNA gene...	329	2e-88
gi|U97055|TSU97055 Theileria sp. 18S ribosomal RNA type G gene,...	321	6e-86
dbj|AB012195|AB012195 Theileria sp. gene for small subunit ribo...	321	6e-86

notes: ① invalidated
② felis
③ muris

gb|AB[illegible]|AB[illegible]	Theileria sp. gene for small subunit ribo...	321	6e-86
gb|L12364|THEIASRRNA	Theileria parva 18S ribosomal RNA (18S rRN...	321	6e-86
gb|AF[illegible]|AF[illegible]	Theileria parva small subunit ribosomal RN...	321	6e-86
gb|L19080|THEPRIBSA	Theileria taurotragi 18S ribosomal RNA.	321	6e-86

gb|U[illegible]|CWRASRRN Cryptosporidium wrairi small subunit ribosomal RNA gene.
Length = 1746

Score = 684 bits (346), Expect = 0.0
Identities = 383/393 (97%), Gaps = 2/393 (0%)
Strand = Plus / Plus

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Sbjct: 622  ttaataatttatatataatattttgaaaatatttatataatattaacataattcatatta 681

Query: 60   ctat-tatttattagtatatgaaatttttactttgagaaaattagagtgcttaaagcaggct 118
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Sbjct: 682  ctatatatttttagtatatgaaatttttactttgagaaaattagagtgcttaaagcaggca 741

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Sbjct: 922  attaatcaagaacgaaagttaggggatcgaagacgatcagataccgtcgtagtcttaacc 981

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gb|AF093492|AF093492 Cryptosporidium parvum strain Human C. parvum genotype (HFL5
subunit ribosomal RNA gene, complete sequence
Length = 1745

Score = 670 bits (338), Expect = 0.0
Identities = 382/393 (97%), Gaps = 4/393 (1%)
Strand = Plus / Plus