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ADDENDUM TO DRAFT DRINKING WATER CRITERIA DOCUMENT FOR *CRYPTOSPORIDIUM*

Addendum to Draft Drinking Water Criteria Document for *Cryptosporidium*

U.S. Environmental Protection Agency
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EXECUTIVE SUMMARY

The Safe Drinking Water Act requires the U.S. Environmental Protection Agency (EPA) to publish regulations to control disease-causing organisms (pathogens) and hazardous chemicals in drinking water. One of the regulations published by EPA to control pathogens is known as the Surface Water Treatment Rule (54 FR 27486: June 29, 1989). The intent of this rule was to control *Giardia*, pathogenic viruses, and *Legionella*, all of which have caused many outbreaks and cases of waterborne illness.

Another prominent waterborne pathogen is the protozoan *Cryptosporidium*. This organism has caused a number of waterborne disease outbreaks in the U.S. and other countries. In 1994, EPA prepared a literature review of the published data on *Cryptosporidium*, entitled "*Cryptosporidium* Criteria Document", to establish a basis for a regulation to control this organism. The following document, " Addendum to Draft Drinking Water Criteria Document for *Cryptosporidium*", updates the 1994 publication. It includes new information available in the literature from 1994 to the present, and was prepared to support EPA's Interim Enhanced Surface Water Treatment Rule, which has as a primary focus the control of *Cryptosporidium*. The update provides information on general characteristics of *Cryptosporidium*, its occurrence in human and animal populations and in water, the health effects associated with *Cryptosporidium* infection, outbreak data, and an assessment of risk. The document also includes information about analytical methods to enumerate *Cryptosporidium* in water and the effectiveness of various water treatment practices.

The document demonstrates that *Cryptosporidium* oocysts are common and widespread in ambient water, and can persist for months in this environment. The dose that can infect humans is low, and a number of waterborne disease outbreaks caused by this protozoan have occurred in the U.S., most notably in Milwaukee, where an estimated 400,000 people became ill. The document shows that otherwise healthy people recover within several weeks after becoming ill, but illness may persist and contribute to death in those whose immune systems have been seriously weakened (e.g., AIDS patients). Drugs effective in preventing or controlling disease are not yet available. The public health concern is aggravated by the resistance of *Cryptosporidium* to commonly used water disinfection practices such as chorine. However, a well-operated water filtration system is capable of removing at least 99 of 100 *Cryptosporidium* oocysts in the water. Monitoring for this organism in water is currently difficult and expensive.

EPA believes that the information in the 1994 document and the following update is sufficient to conclude that *Cryptosporidium* may cause a health problem and occurs in public water supplies at levels which may pose a risk to human health.

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I. Introduction

The United States Environmental Protection Agency (USEPA) Office of Water is preparing and revising the health criteria documents that will support the Phase I Disinfectant/Disinfectant Byproduct (DBP) Rule, the Interim Enhanced Surface Water Treatment Rule (IESWTR) and the Groundwater Disinfection Rule (GWDR). As part of the rule making process, the USEPA is required to compile a complete and current compendium of the information used as criteria to support creation of the rules. The first step in this process occurred in June of 1994 with the preparation of the USEPA Draft Drinking Water Criteria Document for *Cryptosporidium*, hereafter referred to as the "1994 *Cryptosporidium* Criteria Document". This addendum provides an update to supplement (but not duplicate) the 1994 *Cryptosporidium* Criteria Document. This addendum uses the same table of contents formatting as the 1994 document to facilitate cross referencing between the two. Much of the published research since the 1994 document focuses on better methods to detect *Cryptosporidium* in the environment, and improvements in water treatment technology. Consequently this addendum includes much new information regarding improvements in analyses and treatment. The overall objective is to provide a comprehensive *Cryptosporidium* information resource to Federal, State, and local health officials responsible for protecting public health and the environment.

II. General Information and Properties

A. History and Taxonomy

1. History

Despite the discovery of *Cryptosporidium* in a wide variety of animal species, this parasite was considered medically unimportant for almost seventy years after its initial description by Tyzzer in 1907 (Soave, 1995). However, the diagnosis of cryptosporidiosis in humans in 1976 and the subsequent connection of *Cryptosporidium* to epidemic waterborne disease have since fostered worldwide interest in the study of this microorganism. By the time the Centers for Disease Control and Prevention implemented routine reporting for *Cryptosporidium* among AIDS patients in 1982, only 13 cases of human cryptosporidiosis had been documented (Ungar, 1990). Since 1982, more than 1000 reports of human cryptosporidiosis have been documented in almost 100 countries, reaching all continents with the exception of Antarctica (Fayer, 1997). At the time of this writing, it is estimated that the annual number of cryptosporidiosis cases exceeds several million worldwide (Casemore *et al.*, 1997).

Cryptosporidium was first recognized as a waterborne pathogen during an outbreak in Braun Station, Texas, where more than two thousand individuals were afflicted with cryptosporidiosis (D'Antonio *et al.*, 1985; Graczyk *et al.*, 1998b). Since that time, outbreaks affecting over a million individuals have been documented throughout North America and Europe, with the single largest epidemic occurring in Milwaukee, Wisconsin in 1993 (Mackenzie *et al.*, 1994). A complete history of the waterborne outbreaks of cryptosporidiosis is provided in section III-F.

2. Taxonomy

Cryptosporidium is one of several protozoan genera in the phylum Apicomplexa which develop within the gastrointestinal tract of vertebrates throughout their entire life cycles. More than twenty species have been described based upon the hosts from which they were originally isolated (a complete list is included in Table II-1, 1994 *Cryptosporidium* Criteria Document). However, interspecies transmission studies, morphological examination and immunological analyses have reduced this number to eight valid species (Table 1). A number of species isolated from amphibians, reptiles, and birds remain unnamed, pending taxonomic classification (Fayer, 1997). Moreover, the taxonomy of the genus *Cryptosporidium* is uncertain and changing (Tzipori and Griffiths, 1998; Feyer et al., 1997).

Table 1 Valid *Cryptosporidium* Species

Cryptosporidium Species	Host Species
C. baileyi	*Gallus gallus* (domestic chicken)
C. felis	*Felis catis* (domestic cat)
C. meleagridis	*Meleagris gallopavo* (turkey)
C. muris	*Mus musculus* (house mouse)
C. nasorum	*Naso literatus* (fish)
C. parvum	*Mus musculus* (house mouse)
C. serpentis	*Elaphe guttata* (corn snake)
	E. subocularis (rat snake)
	Sanzinia madagasarensus (Madagascar boa)
C. wrairi	*Cavia porcellus* (guinea pig)

Source: Adapted from Fayer (1997) and Upton (1989)

Although infection caused by *Cryptosporidium* has been observed in over 70 mammalian and reptilian hosts, illness in humans is confined primarily to infections associated with *Cryptosporidium parvum*; a single case of human cryptosporidiosis attributed to *C. baileyi* in an immunocompromised individual has been reported (Ditrich, 1991), but this organism was later

shown to be *C. Parvum* (Fayer, pers. Comm.) . Until recently, it has not been possible to assess variation among strains or to evaluate the possibility of distinct transmission cycles among different isolates. Studies utilizing restriction fragment length polymorphism (RFLP) analysis, isoenzyme electrophoresis and arbitrarily primed polymerase chain reaction (AP-PCR) have helped to characterize subtle differences among individual strains and are described in this section.

A study harnessing the polymerase chain reaction and restriction mapping suggests that differences in the genetic sequences within the 18S ribosomal RNA (rRNA) region of *Cryptosporidium* can be used to distinguish individual strains (*muris*, *parvum*, and *baileyi*) and may assist in the development of taxonomic classification (Awad-El-Kariem *et al.*, 1994). Webster (1993) applied a battery of molecular taxonomic methods (flow cytometry, PCR, and RFLP) to detect and classify *Cryptosporidium* oocysts from geographically diverse isolates. Isolates exhibited genetic homogeneity for the most part, however differences in isoelectric points and restriction maps indicated genetic differences among *C. parvum* isolates from humans and bovines.

Isoenzyme electrophoresis studies (O'Donoghue, 1995; Awad-El-Kariem, 1995) have been applied to characterize animal and human oocyst isolates of *Cryptosporidium parvum* from different geographical locations. The discovery of two unique isoenzyme forms indicate the existence of separate subpopulations within the *parvum* strain, one which infects primarily humans and the other which infects animals. Follow-up studies using arbitrary primed

polymerase chain reaction (AP-PCR) and isoenzyme typing (Carraway *et al.*, 1994; Awad-El-Kariem, 1996; Awad-El-Kariem, 1997) have confirmed the two unique 'profiles' among *C. parvum* corresponding to the animal and human types; cross-transmission infection studies performed by Awad-El-Kariem *et al.* (1996) indicated that most human isolates were not capable of establishing infection in a murine model, whereas all animal isolates were infectious in mice, supporting the existence of genetically distinct populations of this strain.

Evidence collected during these studies supports the hypothesis that *Cryptosporidium* may belong to a clonal population structure, based on correlations between phenotypic and genotypic markers, and the widespread occurrence of identical genotypes. The essential concept of the clonal hypothesis is that different strains of the same species may clone (or propagate) different forms of the same gene due to geographic isolation. As a consequence of this isolation, mutated or otherwise modified genetic loci will become perpetuated in a clonal manner within the species in the absence of sexual reproduction. *C. parvum* may be clonally related to *Cryptosporidium* isolated from Guinea Pigs (*C. wrairi*), but it is not yet clear whether they should be considered as two or more species or subspecies.

Analysis of genetic polymorphism among *C. parvum* isolates from nine human outbreaks and from several bovine sources (Peng *et al.*, 1997) indicates the existence of two genotypes (genotypes 1 and 2) with genetic differences among adhesion proteins. Genotype 1 was observed exclusively in human isolates; genotype 2 was observed both in calf isolates and in isolates from human patients who reported direct or indirect exposure to infected cattle, supporting the

occurrence of two distinct transmission cycles of *C. parvum* in humans: (1) human to human, and (2) animal to human. This hypothesis is supported by the work of Carraway *et al.* (1997), who conducted RFLP analyses on *C. parvum* oocysts isolated from humans and cows. While all calf isolates exhibited genetic homogeneity at a specific 2.8-kb fragment, human isolates exhibited multiple profiles at this locus, one found exclusively in humans and one, which indicated a superposition of both profiles, indicative of heterogeneity among parasite populations.

B. Life Cycle

A complete description of the life cycle of *Cryptosporidium* is provided in the 1994 USEPA *Cryptosporidium* Criteria Document (Figure II-1, p. II-5). *Cryptosporidium* is excreted in the feces of an infected host in the form of an oocyst, which represents the only exogenous stage of the life cycle. The oocyst consists of four sporozoites housed within a sturdy, multi-layered wall. The thick-walled oocyst is the environmentally resistant form of this parasite, resulting from the fertilization of macrogametes within the host, and is appreciably resistant to natural decay in the environment as well as to most disinfection processes. The life cycle is repeated when sporulated oocysts excreted by an infected host are ingested by a new host and the sporozoites excyst within the small intestine.

C. Morphological Features

A complete description of the morphological features of each life cycle stage (oocyst, sporozoite, trophozoite, merozoite, microgametocyte, macrogametocyte) of *Cryptosporidium* is provided in

the 1994 *Cryptosporidium* Criteria Document (p.II-7 – II-9). Studies conducted between 1994 and 1998 indicate that the surface "fold" previously thought to be synonymous with the suture described in ultrastructural studies may not be the same (Robertson, 1994), suggesting that the fold should no longer be described as a suture, and that during microscopic examination, objects matching the size, shape and fluorescence criteria should not be discounted based upon the absence of a surface fold pattern.

D. Species Transmission

1. Direct Transmission Between Humans

A number of studies show that person-to-person transmission of cryptosporidiosis infection can occur within families, day care centers, hospitals and in urban environments where population densities are high (1994 *Cryptosporidium* Criteria Document). The route of infection follows one of two paths: direct, through fecal-oral contact, or indirect, through fomites (inanimate objects).

After visitors who came to Milwaukee during the 1993 outbreak returned to their homes, 74 members of their households who had not accompanied them on the visit tested positive for *Cryptosporidium* (Mackenzie *et al.*, 1995b). Five percent of these infected household members ultimately developed cryptosporidiosis, thereby meeting the definition of secondary transmission within the household. Osewe *et al.* (1996) also evaluated data following the Milwaukee outbreak and concluded that secondary transmission rates to household members during the epidemic were comparatively low (3-5%). In addition to the person-to-person transmission, there appeared to

be a recurrence of infections that were acquired by the Milwaukee visitors during the outbreak. Transmission to susceptible individuals in the Milwaukee area continued after the massive initial outbreak, but decreased rapidly within the two months that followed.

Cordell and Addiss (1994) tracked cryptosporidiosis in various child care settings and observed a 12 to 22% rate of secondary spread between children to other household members. Newman *et al.* (1994) reported household transmission of *C. parvum* infection in an urban community in northeast Brazil. In this study, 18 of 31 (58%) households whose members ranged in age from 5 months to 47 years showed at least one secondary case of cryptosporidiosis identified either by stool examination or serologic testing. Secondary cases involved a total of 30 persons, yielding an overall transmission rate of 19%. Of the 202 persons included in this study, 94.6% had evidence of serum IgG or IgM antibodies to *Cryptosporidium*, demonstrating that a significant rate of person-to-person transmission of *Cryptosporidium* may occur.

The rate of transmission between immunocompromised individuals may be high. In group homes housing HIV-positive individuals, Heald and Bartlett (1994) reported a high (not specified) rate of transmission among occupants. Lopez-Velez *et al.* (1995) found an overall prevalence of intestinal cryptosporidiosis of 15.6% in AIDS patients. However, the rate among homosexual partners was higher (33.3%) than among intravenous drug users (10.6%), strongly suggesting person-to-person transmission of cryptosporidiosis. Sears *et al.* (1994) concluded that strict infection control measures must be followed especially in crowded living conditions and where immunocompromised persons reside.

Secondary transmission of cryptosporidiosis has been observed among humans whose occupation places them near primary cases within a confined space. An outbreak occurred among crew members on a U.S. Coast Guard cutter that had obtained water from the city of Milwaukee during the 1993 epidemic (Moss *et al.*, 1994). Of 50 crewmembers, 62% exhibited symptoms of cryptosporidiosis and oocysts were detected in stool samples of 10 individuals (20%). In such an outbreak, distinguishing between primary infections (i.e., due to ingestion of contaminated water) and secondary infections (i.e., due to fecal contaminated fomites, food, or other infected individuals) is difficult because the individuals involved were obviously in a closed environment where frequent contact between humans occurs. In some instances it may not be possible to determine whether transmission between humans is the primary cause of cryptosporidiosis, especially when humans also come into contact with animals through occupational or recreational activities (Adam *et al.*, 1994).

Adegbola *et al.* (1994) reported that the transmission rate of *Cryptosporidium* between children has seasonal peaks associated with rain and high relative humidity. Factors accounting for the seasonal distribution may include increased survival of oocysts in a high relative humidity environment and possible dissemination of oocysts to children during heavy rains as a result of decreased domestic and environmental hygiene.

2. Transmission Between Animals and Humans

The 1994 USEPA *Cryptosporidium* Criteria Document cites adequate evidence for the

transmission of *Cryptosporidium* from animals, particularly livestock, to humans. Of eight valid species infecting all vertebrate groups, only one, *Cryptosporidium parvum*, represents a global public health problem due to its zoonotic potential (Graczyk, 1998b). Other than a single report of *C. baileyi* infection in an immunosuppressed individual (Dítrich, 1991), infections in humans have been confined to *C. parvum*.

A number of studies have been conducted to determine if *C. parvum* can cross species lines to non-mammalian species. Graczyk *et al.* (1996a) attempted to infect a variety of fish, amphibia and reptiles with *C. parvum* without success. Although the oocysts were present in the cloaca of two fish and one lizard post-exposure, no life-cycle stages were detected in histological sections taken from any of the inoculated species. From these results, the authors concluded that the oocysts were unable to establish an infection in the gastrointestinal tract of lower vertebrates even though the inoculated oocysts were retained by these animals at least 14 days following ingestion. Although animal excreta has been shown to contaminate watersheds and source waters linked to outbreaks (see Section III-C), evidence of direct animal transmission of *Cryptosporidium* to humans has been limited to a few examples (Adam, 1994; Casemore, 1990). The search for the source of *Cryptosporidium* in sporadic cases has been just as elusive. To assess infectious risks associated with pet ownership, Glaser *et al.* (1998) conducted a case-control study of HIV-infected individuals with and without cryptosporidiosis. No statistically significant differences were observed in the rate of overall pet ownership, cat ownership, or bird ownership between the two groups, and only a slight correlation between dog ownership and human cryptosporidiosis was noted. The authors concluded that pets do not represent a major

risk factor for acquisition of cryptosporidiosis.

Despite the strict host-specificity of a number of mammalian strains of *Cryptosporidium*, the discovery that a *C. parvum*-refractive host (a host which ingests infectious oocysts but is not susceptible to infection) can excrete intact ingested oocysts has raised the issue that if oocyst infectivity is retained during travel through the intestinal tract, that host could serve as a mechanical vector for dissemination of the parasite through the environment (Graczyk, 1998b). Transport of oocysts through migratory waterfowl has epidemiological implications which should be considered, especially around reservoirs or in waters where shellfish are harvested and may be consumed raw (Fayer *et al.*, 1997; Smith *et al.*, 1993).

E. Summary

Cryptosporidium has a complex life cycle which involves numerous developmental stages culminating in the production of environmentally resistant oocysts. Eight species have been currently classified. The species known to infect humans, *C. parvum*, appears to be infectious to at least 78 other mammals; however, at least one genotype within *C. parvum* appears to be transmitted exclusively through humans. There is sufficient evidence that secondary *Cryptosporidium* infections can occur in humans following primary infection from ingestion of contaminated drinking water. Human-to-human transmission occurs through the fecal-oral route and reinfections are common where human density is high or among people living in close quarters. *C. parvum* exhibits species specificity with regard to cryptosporidiosis. Lower vertebrates such as fish, frogs, and lizards are not susceptible to infection. Other than *C. parvum*,

only the avian species *C. baileyi* has been associated with human cryptosporidiosis. Species that infect other mammals, such as *C. wrairi* may be clonally linked to *C. parvum*; however, the public health significance of this relationship concerning potential transmission of *C. wrairi* among human populations is not known.

III. Occurrence

A. Worldwide Distribution

1. Distribution in Animal Populations

Although the 1994 *Cryptosporidium* Criteria Document acknowledges the fact that members of the genus *Cryptosporidium* have been identified in many animal species, it focuses primarily on the prevalence of *Cryptosporidium* in domestic animals (cattle, lambs, pigs, goats, deer, and horses) and house pets (dogs, cats, hamsters, guinea pigs, and rabbits). Prior to the writing of the 1994 document, and since that time, *Cryptosporidium* has been identified in numerous mammalian, avian, reptilian and piscine hosts worldwide. A recent, frequently cited review article contains an extensive list of the animals in which *Cryptosporidium* has been detected (O'Donoghue, 1995). The findings of the review article are summarized below

> *Cryptosporidium* infections have been recorded in 78 mammalian species. A list of these species is given in Table 6 of the review article (O'Donoghue, 1995). Natural infections (i.e. infections not induced in an experimental setting) have been described in domestic, wild and captive mammals with the majority occurring in farm, zoo, pet and lab animals. Most infections in mammals have been attributed to *Cryptosporidium parvum*, however, some natural infections in mice, rats, cattle, mountain gazelles and a camel have been attributed to *C. muris*.
>
> *Cryptosporidium* infections have been detected in over 30 species of birds primarily in domestic flocks or aviary birds, but also in wild bird populations. Two species of

Cryptosporidium are currently considered as valid pathogens in birds (*C. meleagridis* and *C. baileyi*, See Table 1, section II-A) and infections have been attributed to both, although neither species has demonstrated host specificity.

Cryptosporidium infections have been reported in over 57 different reptilian species including 40 species of snakes, 15 species of lizards, and 2 species of tortoises. *Cryptosporidium serpentis* is currently the only valid named species of *Cryptosporidium* known to cause infections in reptiles. Previous descriptions of other species causing infections in reptiles (*C. crotali, C. lampropeltis, C. ameivae and C. ctenosauris*) are consistent with the morphology of another parasite species (*Sacrocystis* sp.) and therefore are considered invalid (Fayer, 1997).

Cryptosporidium infections have been detected in nine species of fish. Infections have been observed in both freshwater and marine species, and in cultured, captive, ornamental and wild-caught fish. The only valid named species of *Cryptosporidium* causing infection in fish is *C. nasorum*, named after the first reported infection in a tropical marine fish (*Naso lituratus*) (Hoover *et al.*, 1981).

In addition to the mammalian, avian, reptilian and piscine hosts mentioned above, *Cryptosporidium* infections have been identified in two amphibian species, *Ceratophrys ornata* (Bell's horned frog, Crawshaw and Mehren, 1987, cited in O'Donoghue, 1995) and *Limnodynastes tasmaniensis* (spotted grass frog, O'Donoghue and Mirtschin, unpublished, cited in O'Donoghue, 1995), and in one invertebrate species, *Ruditapes decussatus* (Portuguese clam, Azevedo, 1989, cited in O'Donoghue, 1995).

Additional hosts reported in the literature, but not mentioned in the review article, include Fell Pony foals (Scholes *et al.*, 1998), muskrat (Petri *et al.*, 1997), African hedgehog (Graczyk *et al.*, 1998a), dugong (Hill *et al.*, 1997), slow loris, white rhinoceros, Indian elephant and Thorold's deer (Majewska *et al.*, 1997) and iguana (Fitzgerald *et al.*, 1998).

2. Distribution in Human Populations

The distribution of *Cryptosporidium* in human populations is worldwide, occurring in both developed and under-developed countries, urban and rural areas, and in temperate as well as tropical climates (Fayer, 1997; O'Donoghue, 1995). The 1994 *Cryptosporidium* Criteria

Document addresses the worldwide distribution of human cryptosporidiosis in 45 different countries. A recent review article states that since 1982, human cryptosporidiosis has been documented in 95 countries and on every continent except Antarctica (Fayer, 1997). A list of these countries including the 45 listed in the 1994 document is given in Table 2.

Table 2 Geographic Distribution of Human Cryptosporidiosis

Africa	**Asia**	**Central/ South America**	**Europe**	**Pacific**
Algeria	Bangladesh	Argentina	Austria	Australia
Burundi	Belarus	Brazil	Belgium	Malaysia
Cameroon	Cambodia	Colombia	Czechoslovakia	New Zealand
Ethiopia	China	Chile	Denmark	Papua-New Guinea
Gabon	India	Costa Rica	England	Philippines
Ghana	Japan	Ecuador	Finland	Singapore
Guinea	Korea	El Salvador	France	
Guinea-Bissau	Myanmar Republic	Guatemala	Germany	
Ivory Coast	Pakistan	Mexico	Greece	**Caribbean**
Kenya	Russia	Panama	Hungary	Cuba
Liberia	Sri Lanka	Peru	Ireland	Haiti
Mauritania	Taiwan	Uruguay	Italy	Jamaica
Mauritius	Thailand	Venezuela	Lithuania	Puerto Rico
Morocco			Netherlands	St. Lucia
Nigeria		**Middle East**	Poland	Tobago
Rwanda		Egypt	Portugal	Trinidad
South Africa		Iran	Romania	Virgin Islands
Sudan		Israel	Serbia	
Togo		Kuwait	Spain	
Tunisia		Saudi Arabia	Sweden	
Uganda			Switzerland	
Zaire		**North America**	Turkey	
Zambia		Canada	Wales	
Zimbabwe		United States		

Source: Fayer, 1997

Worldwide prevalence rates of human cryptosporidiosis from the most recent compilations of coprologic (stool) and serologic (blood) surveys (through 1991) are included in the 1994 *Cryptosporidium* Criteria Document.

B. Occurrence in Water

1. Surface Water

Since the 1994 criteria document was published, dozens of reports have appeared in the literature further characterizing the occurrence of *Cryptosporidium* in surface waters. Several of these articles adequately summarize this large body of work. Lisle and Rose (1995) reviewed the literature for more than 25 studies involving outbreaks, occurrence, monitoring, and detection, as well as regulatory implications. They reported that between 5.6 and 87.1% of source waters tested, contained 0.003 to 4.74 oocysts/L. They concluded that better methods were needed for oocyst recovery, detection, and treatment. In another major study, LeChevallier and Norton (1995) reported finding oocysts in 60.2% of surface waters tested in the U.S. and Canada. Three companion articles appeared in the September 1997 issue of the Journal of the American Water Works Association summarizing the current state of knowledge of *Cryptosporidium* occurrence in watersheds (Crockett and Haas, 1997), rivers (States *et al.*, 1997) and reservoirs (LeChevallier *et al.*, 1997). All three studies concluded that any surface water is subject to a complex set of watershed characteristics (buffer zones, slope, land use, watershed management, storm water management, sewage treatment practices, sediment types, soil types, vegetation, population density, pathogen sources, best management practices and recreational uses) and watershed processes (precipitation, or snow and ice melt derived runoff, sediment re-suspension, dumping, spills, wastewater treatment plant failures, temperature fluctuations, and algal blooms) that may lead to the presence of oocysts.

2. Ground Water

According to the 1994 *Cryptosporidium* Criteria Document, oocysts are found less frequently in

ground water than in surface water and consequently very few cryptosporidiosis outbreaks have been traced to ground water contamination. Kramer *et al.* (1996), in a U.S. national survey for the presence of *Cryptosporidium* oocysts in drinking water, showed that, of the five outbreaks recorded from 1993 to 1994, two outbreaks resulted from untreated well water. Both of the outbreaks occurred in Washington State, one in 1993, which accounted for 7 cases of human cryptosporidiosis, and one in 1994 accounting for 104 cases (Rose *et al.*, 1997). Hancock *et al.* (1998) recently reported a study of 199 groundwater samples tested for *Cryptosporidium*. They found that 5% of vertical wells, 20% of springs, 50% of infiltration galleries, and 45% of horizontal wells tested were positive for *Cryptosporidium* oocysts. This new data will force environmental microbiologists and regulators to reassess previous assumptions that groundwater is inherently free of parasites.

C. Occurrence in Soil

Most *Cryptosporidium* research has centered on detecting oocysts in either water or biological samples. Limited studies have been performed to ascertain the presence or viability of *Cryptosporidium* in soil. Transport of *Cryptosporidium* oocysts to water from fecal contaminated soil within a watershed during weather events was suggested as the most probable mechanism of source water contamination in several documented outbreaks (Kramer *et al.*, 1996). Mawdsley *et al* (1996) reported on the vertical movement of *Cryptosporidium* oocysts through intact 30 cm soil cores. Transport of oocysts through the soil cores was greater in silty loam and clay loam than in loamy sandy soil, exactly the opposite of what the researchers expected. Research funded by the American Water Works Association Research Foundation,

aimed at creating a better understanding of watersheds and soil oocyst interactions is ongoing.

A new method was recently reported by Anguish and Ghiorse (1997) for examining *Cryptosporidium* oocysts in media other than water. They evaluated a computer-assisted laser-scanning microscope equipped for confocal laser scanning and color video microscopy to examine two agricultural soils, barnyard sediment and a calf fecal sample. The authors concluded that this technique provides a better approach for oocyst identification and enumeration, as well as for *in situ* assessment of cellular activity and viability. This technology could have great utility for detecting *Cryptosporidium* in soil.

D. Occurrence in Air

The 1994 *Cryptosporidium* Criteria Document cited no data to show that *Cryptosporidium* is found in ambient air. A review of the current literature indicates that this continues to be an unresearched area.

E. Occurrence in Food and Beverages

The occurrence of several foodborne outbreaks in recent years has highlighted the role of *Cryptosporidium* as a foodborne pathogen. The presence of *Cryptosporidium* has been documented in raw milk (Badenoch *et al.*, 1990), unpasteurized apple cider (Millard *et al.*, 1994), uncooked meat products (Casemore *et al.*, 1987), and in eight of eight types of fresh, Costa Rica grown produce tested (Monge *et al.*, 1995). Refrigeration does not compromise oocyst viability. The influence of temperature on oocyst survival is discussed in section III.G. Recent studies

evaluating oocyst viability (assessed by vital dye staining) showed greater than 85% of oocysts present in beer and cola made from intentionally contaminated water lost their viability, while only 35% of oocysts in reconstituted infant formula and orange juice lost viability (Friedman *et al.*, 1997). The authors speculate the decreased pH of the carbonated beverages triggers premature excystation

F. Specific Disease Outbreaks

1. Outbreaks Associated with Drinking Water

The 1994 *Cryptosporidium* Criteria Document describes recent waterborne disease outbreaks attributed to *Cryptosporidium*. The 1990/1991 and 1992/1993 MMWR reports on waterborne disease outbreaks prepared by the U. S. Center for Disease Control were not included in the 1994 document(Herwaldt *et al.*, 1991 and Moore *et al.*, 1993). Since 1994, numerous papers have appeared in the literature more completely describing the previous outbreaks and documenting new outbreaks.

Additional research articles have been published specific to the 1993 Milwaukee outbreak. Addiss *et al.* (1996) reported on the effectiveness of point-of-use water treatment devices during the Milwaukee outbreak. They concluded that using sub-micron point-of-use devices may significantly reduce the risk of waterborne cryptosporidiosis. Rodman *et al.* (1997) studied the utility of monitoring sales data on non-prescription antidiarrheal medications to detect enteric disease outbreaks. The information from this study showed that, while the technique would have been useful in detecting the Milwaukee outbreak, the costs incurred in collecting the data and the

absence of increased sales during other outbreaks indicate that this tool will be of limited use in other cities. Turbidity spikes at the Milwaukee Water Treatment Plant correlated strongly with hospital visits for gastrointestinal disease prior to 1993 (Morris *et al.*, 1998), indicating that cryptosporidiosis was occurring in Milwaukee for more than a year before the outbreak. Eisenberg *et al.* (1998) confirmed this finding and further concluded that 85% of the outbreak infections could have been avoided if the smaller outbreak had been detected. Researchers concluded after studying Milwaukee death certificates from before and after the outbreak that waterborne outbreaks of cryptosporidiosis can result in significant mortality, particularly among immunocompromised populations (Hoxie *et al.*, 1997). Fox and Lytle (1996) published a summary article outlining the results of the investigation of the Milwaukee outbreak by the USEPA. Moss *et al.* (1998) reported on an outbreak of Cryptosporidiosis involving more than half of the crewmembers of a Coast Guard cutter which had filled its water tanks with Milwaukee water during March of 1993.

Duke *et al.* (1996) reported an outbreak of cryptosporidiosis in Northumberland, U.K. The source water was a private untreated water supply that appeared to be contaminated by run-off slurry from surrounding fields and/or lamb carcasses found in a collection chamber connected to the water supply. Atherton *et al.* (1995) also reported an outbreak of cryptosporidiosis in northern England which was attributed to failure of the public drinking water system to remove oocysts. The outbreak onset was characteristic of a point source infection occurring during a period of heavy rainfall at the reservoir. Maguire *et al.* (1995) reported on an outbreak in London, U.K., where 44 individuals were confirmed to have *Cryptosporidium* infections

acquired from drinking tap water. Bridgman *et al.* (1995) reported on 47 cases of crytosporidiosis linked to a well water supply in northwestern England.

Leland *et al.* (1993) wrote an article not cited in the 1994 *Cryptosporidium* Criteria Document describing the Jackson County, Oregon outbreak of 1992 from an engineering perspective. In a Florida outbreak, 77% of the counselors and campers at a day camp were infected from a municipal drinking water supply (CDC, 1996). Goldstein *et al.* (1996) reported on an outbreak of cryptosporidiosis in Las Vegas, Nevada, within the Lake Mead watershed area, where 78.2% of the cases occurred in immunocompromised persons and 21.8% of the infected individuals were immunocompetent individuals. The treatment system used state-of-the-art technologies and chemical treatment. Recognition of the outbreak was attributed to surveillance conducted by the State of Nevada, where cryptosporidiosis is a reportable disease.

A number of overview articles concerning *Cryptosporidium* outbreaks have been published since 1994. Rose *et al.* (1997) briefly described a number of outbreaks associated with drinking water, and Solo-Gabriele and Meumeister (1996) presented an overview of U.S. outbreaks, supporting their view that current practices and regulations are inadequate to protect consumers from waterborne disease. The MMWR Surveillance for Waterborne Disease Outbreaks for 1993-1994 (CDC, 1996) lists 10 of 29 outbreaks associated with the protozoans *Giardia* and *Cryptosporidium*. A national survey over a 2-year test period (1993 and 1994) identified five outbreaks resulting in 403,271 cases involving *Cryptosporidium* oocysts in drinking water (Kramer *et al.*, 1996). Of this total, 403,000 were from the outbreak in Milwaukee, Wisconsin,

103 were from Las Vegas, Nevada, and 27 were from an outbreak at a resort in Minnesota. All three outbreaks were attributed to surface water as the source. The remaining two outbreaks resulted from contaminated ground water: one from a private well in Washington State (resulting in seven cases), and the other from a community well, also in Washington State (resulting in 104 cases). Notable outbreaks are summarized in Table 3.

Table 3. Outbreaks of Cryptosporidiosis Associated with Drinking Water

Year	State	Number of Cases	Source	Deficiency
1984	Texas	2006	Groundwater(C)	Sewage contamination
1986	New Mexico	78	Surface water(C)	Untreated
1987	Georgia	12,960	River(C)	Treatment deficiency
1991	Pennsylvania	551	Groundwater(C)	Treatment deficiency
1992	Oregon	15,000	Spring/river(C)	Treatment deficiency
1993	Wisconsin	403,000	Lake(C)	Treatment deficiency
1993	Washington	7	Well(I)	Surface contamination
1993	Minnesota	27	Lake(NC)	Unknown
1993	Nevada	103	Lake(C)	Inadequate filtration
1994	Washington	104	Well(C)	Sewage contamination
1995	Florida	72	Not applicable	Cross connection

**NC = Non-Community; C = Community; I = Individual

In addition to the outbreaks described in the literature, a newsletter called *Cryptosporidium Capsule* has anecdotal information about suspected outbreaks in drinking water in Devon U.K., 1995, drinking water in Maine, 1995, drinking water in Collingwood Ontario, 1996, and lakes in Cranbrook and Kelowna BC, 1996, and a groundwater outbreak in the U. K. in 1997.

Two articles have been written about the inadequacies of current surveillance practices in detecting and preventing cryptosporidiosis from drinking water. Craun *et al.* (1997) point out that the coliform test can no longer be used as the sole indicator of a water's microbiological

safety. Frost *et al.* (1996) focused on the importance of epidemiological surveillance and collaboration between water purveyors and community public health departments.

2. Outbreaks Associated with Recreational Waters

Fourteen outbreaks of gastroenteritis related to recreational waters were reported by nine states during 1993 and 1994 (Kramer *et al.*, 1996). Ten of these outbreaks were caused by *Cryptosporidium* or *Giardia*, with five specifically linked to *Cryptosporidium*. Three of the *Cryptosporidium* outbreaks were associated with motel swimming pools; two were associated with community swimming pools. All five pools were filtered or chlorinated, and one had a malfunctioning filter. None of the other pools had identifiable treatment deficiencies. The inability of chlorine levels normally used in swimming pools to kill *Cryptosporidium*, coupled with poor pool filtration equipment maintenance practices, have been suggested as the primary cause of swimming pool related cryptosporidiosis.

Outbreaks associated with recreational waters can be difficult to recognize because the individuals using such facilities may reside in widely separated geographical areas. Forty-four individuals contracted cryptosporidiosis after swimming in a Los Angeles pool where an accidental fecal release occurred (CDC, 1990; Sorvillo *et al.*, 1992). McAnulty *et al.* (1994) reported a community-wide outbreak of cryptosporidiosis in Lane County, Oregon that was linked to swimming at a wave pool with inadequate sand filtration equipment. MacKenzie *et al.* (1995a) reported on a swimming pool related outbreak involving 51 individuals. The outbreak

occurred in a city 75 miles from Milwaukee about 30 days after the massive Milwaukee outbreak. The authors suggest that increased attention should be given to preventing swimming pool related outbreaks following outbreaks of cryptosporidiosis associated with water supplies. Cryptosporidiosis associated with two pools in Dane County, Wisconsin was reported during the summer of 1993 (CDC, 1994). Kramer *et al.* (1998) reported on an outbreak with 38 infected individuals who contracted cryptosporidiosis while swimming in a recreational lake. They speculated that contamination of the lake came from either infected swimmers or run-off.

3. Foodborne Outbreaks

Foodborne transmission of cryptosporidiosis has only rarely been reported. In October 1993, an outbreak of cryptosporidiosis occurred among students and staff who consumed contaminated apple cider while attending an agricultural fair in central Maine. This was the first large outbreak in which foodborne *Cryptosporidium* could be identified and documented as the causative agent (Millard et. al., 1994). A survey, completed for 611 (81%) of the estimated 759 attendees at the fair, found 160 (26%) had primary cryptosporidiosis. *Cryptosporidium* oocysts were detected in the stools of 50 (89%) of the primary and secondary case subjects tested. Oocysts were detected in the apple cider, on the cider press, and in the stool specimen of a calf on the farm of the supplier of the apples used to make the cider. The outbreak underscores the need for agricultural producers to take precautions to avoid contamination of foodstuffs by infectious agents commonly present in the farm environment. The Minnesota Department of Health reported on cryptosporidiosis in 50 attendees of a social gathering who ate a salad contaminated during preparation by a day-care worker (CDC, 1996). Laberge *et al.* (1996) have prepared a review

article in which they list foods associated with *Cryptosporidium* infections that includes; unpasteurized milk, sausage, raw beef, kefir, pelleted feed, silage, powdered milk, raw tripe, and apple cider. Casemore (1990) referred to sausage, offal, and raw milk contamination by *Cryptosporidium*, but did not link these foods to specific outbreaks. Harp *et al.* (1996) reported that standard commercial pasteurization techniques kill 100% of *C. parvum* oocysts. Methods to detect oocysts in food have not been optimized.

4. Outbreaks Among Travelers

The 1994 *Cryptosporidium* Criteria Document stated that cryptosporidiosis has emerged as an important cause of traveler's diarrhea and indicated that illnesses frequently occur in travelers visiting second and third world countries. However, reports since that time indicate that travelers within developed countries such as the U.S. have also acquired *Cryptosporidium* infections.

During the Milwaukee outbreak in 1993, Milwaukee visitors became infected with *Cryptosporidium* as a consequence of drinking water in that city. In addition, upon returning home, they transmitted this parasite to members of their households (MacKenzie *et al.*, 1995b).

5. Outbreaks at Day-Care Centers and Prevalence of Cryptosporidiosis Among Children

The 1994 *Cryptosporidium* Criteria Document discusses the high prevalence of cryptosporidiosis in children and notes that the evidence comes primarily from reports of diarrhea in day-care centers. Cryptosporidiosis is now recognized as a significant disease in childcare settings (Cordell and Addis, 1994). Additional data on the effectiveness of prevention and control

strategies, as well as the economic impact of these outbreaks on the community, state, and country, should be collected. There is evidence that cryptosporidiosis affects children in other countries. In China and Brazil, 42 to 58% of a cross section of children less than 16 years of age were serologically positive for *Cryptosporidium*. Among a similar group in Virginia, less than 17% tested positive. The data from the review by Cordell and Addis (1994) indicate that in impoverished communities, this parasitic infection is highly endemic and occurs in early childhood. Single stool samples from 1,000 apparently healthy children (ages 6 through 14) in Jordan showed that 4% of the children had oocysts and, among these children, 37% were symptomatic (Nimri and Batchoun, 1994). Brandonisio *et al.* (1996) reported 7 (1.9%) of 368 children hospitalized (for unspecified reasons) in Italy tested positive for oocysts (359 of these children were immunocompetent and nine were HIV-infected). Six of the seven children were immuno-compromised. Brannan *et al.* (1996) found that 12% of the children in Romania were carrying *C. parvum* oocysts, although 73% of these children had either IgA or IgG antibodies to this protozoal parasite.

6. Sensitive (Immunocompromised) Populations

Immunocompromised populations are at high risk of infection and disease from drinking water contaminated with *Cryptosporidium* oocysts. Clayton *et al.* (1994) studied 41 patients with AIDS who had become infected with *Cryptosporidium* and found two significant patterns among these individuals. In 61% of these patients, *Cryptosporidia* were present in the proximal small bowel (i.e., upper small intestine) and they had severe clinical disease characterized by malabsorption of nutrients. In the remaining 39% who had less severe disease, *Cryptosporidium* was seen only

in the colon or the stool. An increased susceptibility to cryptosporidiosis in immunocompromised individuals was reported in Kenya, East Africa where 42 to 44% of the HIV-positive patients at the Kenyatta National Hospital tested positive for *Cryptosporidium*, whereas only 8.6% of the non-AIDS patients carried this pathogen. In Zambia, 25.4% of the HIV-positive patients who were symptomatic carried oocysts and had increased specific anti-*Cryptosporidium* IgG and IgA antibodies, but there was no increase observed in specific IgM antibodies (Cevallos *et al.*, 1995). This antibody profile suggests that the infection was of prolonged duration.

The cryptosporidiosis outbreak in Las Vegas, Nevada, (Roefer *et al.*, 1995) was recognized primarily because of the high rate of incidence among the immunocompromised population and because of state requirements for reporting of disease from this pathogen. Although the water supply in this city was processed by a state-of-the-art system, the protection it provided proved inadequate for the immunocompromised person. Research shows HIV-infected patients who have cleared oocyst infections have much higher levels of specific secretory IgA levels than AIDS patients with chronic cryptosporidiosis (Flanigan, 1994). This indicates either that secretory IgA is involved in recovery from infection, or that it is the only marker for an effective immune response at the mucosal surface.

In a study on asymptomatic carriage of intestinal *Cryptosporidium* by immunocompetent and immunodeficient children, the percentage of carriers among immunodeficient children was 22% as compared to 6.4% among immunocompetent children (Pettoello-Mantovani *et al.*, 1995).

However, the percentages of symptomatic children in these groups were more similar, with 4.4% of the immunocompetent children and 4.8% of the immunodeficient children being positive for oocysts, respectively.

Greenberg *et al.* (1996) reported on stool sampling from AIDS patients receiving chemotherapy and/or radiotherapy. The study objective was to determine the yield of *Cryptosporidium* oocysts in stools versus biopsies taken from the upper and lower intestines of these patients. Only 53% of 106 patients were positive for oocysts when a single stool sample was taken, but detection increased to 73% positives when multiple stool samples were taken. When sampling was by terminal ileum biopsy, the number of positives increased to 91%. From these studies, it appears oocysts may invade the small intestine in immunocompromised individuals with no oocysts detectable in stool examinations. Thus, stool sampling can be expected to miss a substantial number of *Cryptosporidium* infections in immunocompromised/AIDS patients.

In addition to gastrointestinal disease, AIDS patients can have other complications from *Cryptosporidium* infection such as respiratory cryptosporidiosis (Mifsud *et al.*, 1994). Clavel *et al.* (1996a) reported cases of intestinal cryptosporidiosis with pulmonary involvement in AIDS patients who had diarrhea and were positive for *Cryptosporidium* oocysts.

Patients with cancer may be immunocompromised as a side effect of their therapeutic treatment. Therefore, these patients are likely to have an increased susceptibility to cryptosporidiosis. Tanyuksel *et al.* (1995) examined 106 cancer patients, all of whom were receiving chemotherapy

and/or radiotherapy and surgery, and found that 17% of these patients who had diarrhea were positive for *Cryptosporidium* oocysts. They concluded that individuals who are compromised by such treatment are a high risk for *Cryptosporidium* infections.

Logar *et al.* (1996) evaluated the occurrence of *C. parvum* in Slovenia and reported a higher incidence of cryptosporidiosis in older patients and in young children. The authors believed that the infections in older patients and young children (median age 3 years) were due to lowered resistance or immune response. In the older patients, *Cryptosporidium* infections appeared to be a consequence of other diseases or secondary to irradiation or other immunocompromising treatments common in this age group. Considerable evidence exists to show that immunological deficiency is a natural consequence of aging (Miller, 1996).

G. Environmental Factors

Because *Cryptosporidium* oocysts are remarkably resistant to inactivation in the environment, the survival of *Cryptosporidium* under a variety of environmental conditions has been evaluated by a number of investigators. While the majority of these studies have considered the effects of physical antagonism (freezing, heating, shearing, pressure, UV exposure, etc.), studies have also been conducted to consider the role of microbial antagonists (microbial predation), chemical antagonists (such as disinfection) and aging. At least two studies have considered various combinations of environmental antagonists (shearing *and* disinfection, etc.). This section will focus primarily on aspects of physical antagonists in the environment. The aspects of chemical disinfectants will be covered under Section 4 – "Inactivation of *Cryptosporidium* Under Water

Treatment Practices".

Robertson *et al.* (1992) evaluated the sensitivity of *C. parvum* oocysts to the freezing conditions that occur in cold climatic regions or during the winter season. More than 97% of the test oocysts were inactivated after incubation at –22°C for 18 days, suggesting that the levels of viable oocysts in surface waters subjected to freezing might be influenced by seasonal temperature variations. Cryopreservation studies conducted by Fayer *et al.* (1991) and Fayer (1997) indicate that oocyst survival depends upon the temperature and duration of freezing conditions, implying that *C. parvum* oocysts are not necessarily rendered noninfectious by being frozen. Temperature stability studies were also conducted by Sattar *et al.* (in press, 1998) who evaluated the freeze/thaw susceptibility of various preparations of oocysts including highly purified preparations as well as infected calf feces. This study indicated that oocyst stability under freezing conditions is at least partially dependent upon the surrounding matrix, with fecal material conferring a cryopreservative effect on oocysts. In the absence of freezing conditions, colder water temperatures tend to promote the survival of most microorganisms. *C. parvum* may survive outside of the mammalian hosts for several months or more depending upon water temperature (Straub *et al.*, 1994). Under conditions of high temperatures, Fayer (1994) indicated that all evidence of *C. parvum* infectivity was lost within 60 seconds when temperatures exceeded 72°C or when temperatures of at least 64°C were maintained for two minutes.

Limited studies have been conducted to evaluate the effects of physical shear on oocyst viability to assess the potentially abrasive effects of oocyst contact with sand and gravel particles or

through fast-flowing waters. Parker and Smith (1993) demonstrated rapid inactivation of oocysts in a mixed sand reactor. Sattar *et al.* (1998) conducted studies to evaluate synergistic effects of mixed sand reactors upon disinfection efficiency with chlorine, and observed that shear stress enhanced chlorine inactivation. Sattar *et al.* (1998, in press) also evaluated the effects of microbial predation upon oocyst survival and observed that oocysts incubated in dialysis cassettes suspended in natural waters exhibited significantly longer survival times when bacterial populations were excluded from the suspension water, implying that microbial predation may play an important role in determining oocyst survival in natural waters.

H. Summary

In summary, cryptosporidiosis is zoonotic, widespread and often associated with surface water, groundwater, recreational water, and contaminated food and drink. Outbreaks associated with these sources continue to occur among travelers, children in day-care centers, and immunocompromised as well as healthy individuals throughout the world. Eight valid species of *Cryptosporidia* have been described in 78 mammalian species, 30+ avian species, 57 reptilian species, nine species of fish and two amphibians. Until more research is completed, public health workers can do little more than speculate on the human infectivity of all the *Cryptosporidium* species. Also, there is also little information currently published on the cross-reactivity of the nine species to commercially available antibodies used for detection of the organism. Immuno-compromised individuals such as those with HIV infections or AIDS, very young children, the elderly, and individuals undergoing therapeutic treatment for cancer are more likely to acquire an infection, develop cryptosporidiosis, and show more severe clinical

symptoms. Deficiencies in water treatment systems are often cited as a major reason for outbreaks, and even the best of systems can be overwhelmed by a high density of oocysts entering the source waters over a short period of time. Infected individuals will shed oocysts in their feces and can be expected to transmit the infection to other family or community members. In addition, day care centers for children, because of their high density of a sensitive population, are a potential source for secondary spread of cryptosporidiosis from infected children to others both within and outside of their households. Research on environmental factors confirms previous work showing that oocysts are highly refractile to environmental stressors.

IV. Health Effects in Animals

A. Symptomatology and Clinical Features

The 1994 *Cryptosporidium* Criteria Document includes general information on the symptomology and clinical features of cryptosporidiosis in a limited number of domesticated mammals such as calves and lambs. Cryptosporidiosis has also been documented in many additional mammals, both domestic and wild, as well as several species of birds, reptiles, and fish (see section III-A). Two recent review articles contain comprehensive information on the symptomology and clinical features of cryptosporidiosis in these different groups of animals. (Fayer, 1997; O'Donoghue, 1995).

In general, the development of *Cryptosporidium* depends on the species, age and immune status of the host (Fayer, 1997). Younger animals and animals with less developed or compromised immune systems are generally more susceptible to severe infection than healthy adult animals.

In many cases, infected, healthy adult animals are asymptomatic or possess mild clinical signs (O'Donoghue, 1995). A summary of the symptomology and primary clinical features of infected animals follows.

Mammals: Most clinical cases of cryptosporidiosis in mammals involve infection by *C. parvum*. The most common feature of cryptosporidiosis in mammals is profuse watery diarrhea that may be pale yellow in color and may have a distinct offensive smell. Other clinical signs of infection include: dehydration, fever, anorexia, weight loss, weakness and progressive loss of condition (O'Donoghue, 1995). Most animals will recover spontaneously within 1-2 weeks of infection. Histopathological observations may reveal several lesions in the small intestine including mild to moderate villous atrophy and loss of epithelial cells. Developmental stages of the parasite are often seen within the small intestine and occasionally elsewhere (stomach, colon, liver, lungs) (O'Donoghue, 1995). Fayer (1997) contains detailed descriptions of the symptomology and clinical features of cryptosporidiosis in several species of ruminant and non-ruminant mammals.

Birds: Two *Cryptosporidium* species, *C. meleagridis* and *C. baileyi*, are known to cause infection in birds. Avian cryptosporidiosis appears as either respiratory, enteric, or renal disease (Fayer, 1997; O'Donoghue, 1995). Normally, only one condition manifests during an outbreak, and respiratory infections are more common than both enteric and renal infections (Fayer, 1997, O'Donoghue, 1995). Clinical signs of respiratory infections include rales, coughing, convulsive sneezing, and dyspnea (O'Donoghue, 1995). Excess mucus may exist in the trachea, sinuses and nasal passages, and fluid may be present in the air sac (Fayer, 1997). Histopathological changes

may include hypertrophy and hyperplasia of the respiratory epithelium with reduced or absent ciliation (O'Donoghue, 1995). Parasites may be detected throughout the respiratory tract including the nasopharynx, larynx, trachea and bronchi (O'Donoghue, 1995).

Clinical signs associated with enteric infections caused by cryptosporidiosis in birds include mild to severe diarrhea, dehydration, depression, weight loss and weakness. Histopathology may reveal atrophy and fusion of villi along with epithelial hyperplasia and hypertrophy and other malformations (O'Donoghue, 1995). Parasites may be found primarily in the gastrointestinal tract (O'Donoghue, 1995).

Renal infections in birds have been detected at necropsy only (O'Donoghue, 1995; Fayer, 1997). In these cases, the kidneys were pale in color (Fayer, 1997) and enlarged (O'Donoghue, 1995). Hypertrophic and hyperplastic epithelial cells were detected throughout the kidney (O'Donoghue, 1995; Fayer, 1997).

Reptiles: *Cryptosporidium serpentis* is the only valid, named species of *Cryptosporidium* causing infection in reptiles, although up to five species may exist based on oocyst morphology (Fayer, 1997). Most reports of infections in reptiles have involved clinical or subclinical infections in captive reptiles (especially snakes); only subclinical infections have been reported in wild reptiles (Fayer, 1997). Cryptosporidiosis in snakes is characterized by anorexia, regurgitation, lethargy, firm midbody swelling, weight loss and death (Fayer, 1997). Interestingly, most infections in snakes have been detected in mature animals. In addition, most

infections have been associated with chronic gastric disease as opposed to acute enteritis common in mammals. Infections and intermittent oocyst shedding in snakes may last for several months to two years (O'Donoghue, 1995). Histopathological observations have included inflammation, hyperplasia and hypertrophy of the gastric glands (O'Donoghue, 1995).

Clinical features of cryptosporidiosis in lizards have involved primarily subclinical gastric infections, while cryptosporidiosis in tortoises involved gastritis and regurgitation, or progressive wasting (O'Donoghue, 1995).

Fish: Except for the original report of cryptosporidiosis in fish (Hoover *et al.*, 1981), infections in fish have not been associated with clinical symptoms. The original report described a progressive illness in a tropical marine fish (*Naso lituratus*) characterized by anorexia, emaciation, regurgitation and passage of feces containing undigested food. Although developmental stages of the parasite were found attached to the intestinal mucosa, no pathogenic changes were evident in this fish. Since then, parasites have been detected in the intestines or stomach of other fish, but few histopathological changes have been described (O'Donoghue, 1995).

B. Therapy

Treatment of cryptosporidiosis in animals involves a combination of prophylactic and chemotherapuetic drugs along with other preventative measures. As stated in the 1994 *Cryptosporidium* Criteria Document, there is no approved effective treatment for

cryptosporidiosis in animals. However, numerous drugs have been tested in studies that focused both on the treatment of naturally acquired infections, and the treatment or prophylaxis of experimentally induced infections in animals. The majority of efficacy evaluations of agents tested in animals have involved prophylactic as opposed to therapeutic drug regimens (Blagburn and Soave, 1997).

A recent review article lists the numerous anticryptosporidial drugs that have been evaluated in animals (Blagburn and Soave, 1997). The findings of the review article are summarized below.

> Most studies have been conducted in laboratory rodents including mice, rats and hamsters. Over 30 compounds have proven to be effective against cryptosporidiosis in rodents including maduramicin, alborixin, lasalocid, and salinomycin. In some cases, efficacies of the drugs tested exceeded 90% compared to control (nontreated) animals.
>
> Several anticryptosporidial drugs have also been tested in ruminants. Among the drugs demonstrating activity against *Cryptosporidium parvum* infections are paromomycin, lasalocid, halofuginone, and sulfaquinoxaline.
>
> Anticryptosporidial drugs have also been tested in several other types of animals including several species of snakes, birds, and reptiles, as well as mammals (pigs and cats). There has been little success in identifying successful drugs in these animals, however some success was achieved in treating cryptosporidiosis in snakes.

Prevention of cryptosporidiosis in animals is best achieved by eliminating contact with viable oocysts. This is particularly difficult in settings with large numbers of animals such as farms or zoos (Blagburn and Soave, 1997). To prevent infections in these types of settings, infected animals should be quarantined in facilities that can be cleaned and disinfected, contaminated articles and clothing of animal care workers should be cleaned thoroughly or discarded, clean food and water should be provided, and rodents and other wild animals should be restricted access (Blagburn and Soave, 1997). Prevention can also be enhanced by ensuring that neonatal

mammals receive adequate amounts of colostrum early in life (Blagburn and Soave, 1997).

Treatment of animals suffering from cryptosporidiosis is similar to that in humans. Namely, rehydration with fluids and electrolytes along with antidiarrheal drugs and attempted chemotherapy with anticryptosporidial drugs (Blagburn and Soave, 1997).

C. Epidemiological Data

The majority of epidemiological data for cryptosporidiosis in animals are confined to economically important livestock, especially ruminants (Casemore *et al.*, 1997). Three extensive reviews exist which describe the epidemiological data available for animals including both domestic and wild animals. These reviews include information on the prevalence and spread of cryptosporidiosis in many groups of animals including cattle, sheep, goats, pigs, horses, dogs, cats, deer, mice and several other small mammals, as well as many species of birds, reptiles, and fish (Casemore *et al.*, 1997; Fayer, 1997; O'Donoghue, 1995).

In general, clinical infections are seen primarily in neonates and immunocompromised animals. Age–related resistance has been documented in several species and the age of an animal upon infection can greatly alter the severity of the infection, and the prepatent period (Casemore *et al.*, 1997). Adult animals often appear asymptomatic even when shedding small numbers of oocysts (Casemore *et al.*, 1997; Fayer, 1997; O'Donoghue, 1995). Serologic surveys in animals tend to suggest much higher prevalence rates of cryptosporidiosis, especially in adults (Casemore *et al.*, 1997; Fayer, 1997). This prevalence may be due to cross-reaction of other antibodies present in

the sera rather than from actual infections (Casemore *et al.*, 1997).

Potential sources of infection in animals include other infected animals of the same or different species (e.g. it is believed that rodents can infect calves or cattle with *C. parvum*), mechanical carriers such as insects, birds and humans, contaminated feed and water, and other contaminated fomites (inanimate objects) such as bedding, brushes, shovels, and feed utensils (Fayer, 1997).

Additional epidemiological studies reported in the literature, but not mentioned in the review articles, describe the prevalence of cryptosporidiosis in the following animals:

- cattle/calves (Fayer *et al.*, 1998; Olson *et al.*, 1997; Perez *et al.*, 1998; Pena *et al.*, 1997)
- horses (Scholes *et al.*, 1998; Bray *et al.*, 1998; Forde *et al.*, 1998; Johnson *et al.*, 1997)
- lambs (Bukhari and Smith, 1997)
- goats (Koudela and Jiri, 1997; Goyena *et al.*, 1997)
- nonhuman primates (Muriuki *et al.*, 1997; Majewska *et al.*, 1997)
- rodents (Bajer *et al.*, 1997; Bull *et al.*, 1998)
- chickens (Sreter *et al.*, 1996)
- ostriches (Jardine and Verwoerd, 1997)
- pigeons (Rodriguez *et al.*, 1997)
- catfish (Muench and White, 1997)
- muskrat (Petri *et al.*, 1997),
- African hedgehog (Graczyk *et al.*, 1998a),
- dugong (Hill *et al.*, 1997),
- deer (Majewska *et al.*, 1997),
- slow loris (Majewska *et al.*, 1997)
- white rhinoceros (Majewska *et al.*, 1997)
- Indian elephant (Majewska *et al.*, 1997)
- iguana (Fitzgerald *et al.*, 1998).

D. Summary

Cryptosporidium infections have been documented in many different species of mammals, as well as in several species of birds, reptiles and fish. In general, the severity of the infection

depends on the species, age, and immune status of the host. Clinical infections are primarily seen in younger animals and animals with compromised immune systems, while infected healthy adult animals may be asymptomatic or possess mild clinical signs.

Most clinical cases of cryptosporidiosis in mammals involve infections by *C. parvum*. The most common features of cryptosporidiosis in mammals are profuse, watery, diarrhea; dehydration; fever; anorexia and weight loss. Two species of *Cryptosporidium*, *C. meleagridis* and *C. baileyi*, are known to cause infections in birds. Cryptosporidiosis in birds is characterized by respiratory, enteric, or renal infections. Respiratory infections, causing rales, coughing, convulsive sneezing and diarrhea, and enteric infections, causing dehydration, weight loss and weakness, are the most common. The majority of cryptosporidiosis infections in reptiles have been reported in captive snakes. These infections, caused by *C. serpentis*, are characterized by anorexia, post prandial regurgitation, lethargy and midbody swelling. The only clinical infection described in fish was caused by *C. nasorum*, and was characterized by anorexia, emaciation, regurgitation and passage of feces with undigested food.

Treatment of cryptosporidiosis in animals involves a combination of prophylactic and chemotherapuetic drugs along with other preventative measures. Although there is no approved, effective treatment for cryptosporidiosis in animals, several drugs have been tested in rodent and bovine models and have shown substantial success. Several drugs have also been tested in reptiles and birds with limited success. Prevention of cryptosporidiosis in animals is best achieved by eliminating contact with viable oocysts as much as possible. This involves isolation

of infected animals and disinfection of all articles that come into contact with the infected animals.

The majority of epidemiological data for cryptosporidiosis in animals are confined to economically important livestock, especially cattle. There is also information available on cattle, sheep, goats, pigs, horses, dogs, cats, deer, mice and several other small mammals. These studies show that the age of an animal can greatly alter the severity of the infection and the prepatent period, and that the primary sources of infection for animals are other infected animals, mechanical carriers, contaminated feed and water, and other contaminated objects.

V. Health Effects in Humans

A. Symptomatology and Clinical Features

The clinical manifestations of cryptosporidiosis in humans are directly related to the immunocompetence of the host, and may include profuse, nonbloody, watery diarrhea that generally resolves spontaneously within around 48 hours, however variability in clinical symptoms is appreciable. Diarrheal symptoms are generally not distinguishable from those caused by other common enteric pathogens. Other symptoms reported by individuals afflicted with cryptosporidiosis include abdominal cramps, vomiting, lethargy and general malaise. Diarrhea results from a combination of enterocyte damage and physical blockage of the intestinal villi, leading to a disruption in the normal balance of intestinal absorption and secretion (Clark and Sears, 1996). The incubation period in humans is estimated to vary between 2 to 10 days (Arrowood, 1997), with a mean incubation of approximately 7-9 days (Juranek, 1998).

Symptoms generally last for 10-14 days in immunocompetent individuals; diarrhea may persist for several weeks beyond the clearing of other symptoms. Severe protracted illness may result in immunocompromised individuals, with mortality rates which may exceed 50% (Kramer, 1996).

Human volunteer studies have been conducted to assess the infectivity and dose-response of *C. parvum* in humans (DuPont *et al.*, 1995). Sixty two percent of subjects who ingested doses of *Cryptosporidium* ranging from 30 oocysts to 1 million oocysts acquired infection. The infectious dose causing disease in 50% of the population (ID_{50}) for the Iowa strain of *C. parvum* was calculated at 132 oocysts in humans compared with an ID_{50} of 60 oocysts in neonatal mice; however, the test strain of *C. parvum* in this case was adapted to a mouse model prior to challenge studies, which may account for the disparity in ID_{50} values. The mean and median incubation periods for cryptosporidiosis in the study were 9.0 and 6.5 days, respectively. Infected humans developed clinical enteric symptoms that were associated with excretion of oocysts, although one of the eleven subjects who did not pass oocysts passed a single soft stool on day 10 and exhibited enteric symptoms on days 23 through 31. Symptoms of clinical illness included abdominal pains, cramps, and diarrhea in six subjects; six had nausea; one reported vomiting; and one had moderate dehydration. Table 4 summarizes the dosing data and related infection rates from the study. Note that volunteers exhibiting enteric symptoms (diarrhea, loss of appetite, etc.) did not in all cases test positive for cryptosporidiosis.

Table 4. Rate of infection and enteric symptoms as a function of intended dosage

Intended dose	No. subjects	No. (%) Infected		No. (%) with enteric symptoms		No. (%) with cryptosporidiosis	
30	5	1	(20)	0		0	
100	8	3	(37.5)	3	(37.5)	3	(37.5)
300	3	2	(66.7)	0		0	
500	6	5	(83.3)	3	(50)	2	(33.3)
?1000†	7	7	(100)	5	(71.4)	2	(28.6)
Total	29	18	-	11		7	

*Linear regression analysis of the data yielded an r^2 of 0.983 and an ID_{50} of 132 oocysts.
Source: DuPont *et al.* (1995)

Follow-up studies indicate that the number of excreted oocysts and the pattern and duration of shedding vary widely among immunocompetent individuals (Chappell, *et al.*, 1996). In the volunteer challenge study, high variability in shedding patterns was observed, and oocysts were observed intermittently in consecutive stool samples, implying that production of oocysts is not uniform and may be influenced by unknown factors. These data may in part account for the observation that fewer than half of the individuals who acquire illness during an epidemic produce stools positive for *Cryptosporidium* when single samples are submitted for diagnostic analysis.

B. Epidemiological Data

Although only 13 cases of cryptosporidiosis had been documented by the CDC in 1982, human cryptosporidiosis has been reported in almost 100 countries spanning the globe since that time (Ungar, 1990). Because *C. parvum* is ubiquitous, infects most mammals and is highly infectious, all human populations are at risk to some degree (Griffiths, 1998). Screening of select human populations was initiated in the United States during the 1980's, with special emphasis on

children and immune suppressed individuals; however, determining the true prevalence of cryptosporidiosis has proven challenging due to the fact that diagnostic methods exhibit limited sensitivity, and since the majority of individuals who experience mild to moderate diarrheal illness do not generally seek the services of a physician (Juranek, 1998).

In 1994, a workshop was organized by the National Center for Infectious Diseases (NCID) and the United States Environmental Protection Agency (USEPA) to assist the Centers for Disease Control and Prevention and state public health departments in providing guidance on public health issues relating to waterborne cryptosporidiosis (Juranek *et al.*, 1995; Addiss *et al.*, 1995). The workshop, titled "Prevention and Control of Waterborne Cryptosporidiosis: An Emerging Public Health Threat", addressed the following topics: surveillance systems and epidemiological study designs, public health responses, cryptosporidiosis in immunocompromised individuals, water sampling methods and interpretation of results. The recommended approaches to surveillance include the following:

- Making cryptosporidiosis incidents or outbreaks reportable to CDC;
- Monitoring sales of antidiarrheal medication through local pharmacies (also described by Rodman *et al.*, 1997);
- Monitoring logs maintained by health maintenance organizations (HMOs) and hospitals for complaints of diarrheal illness;
- Monitoring the incidence of diarrhea in nursing homes (also described by Proctor *et al.*, 1998);
- Monitoring laboratory data for *Cryptosporidium* (also described by Proctor *et al.*, 1998);
- Evaluating distribution system design in selected cities; and
- Providing prompt epidemiological assistance during outbreaks.

A cohort approach was recommended to facilitate epidemiological study of outbreak data, in which blood tests are performed quickly to screen out negative subjects. Cohort analysis would

demonstrate whether the exposure(s) was associated with subsequent infection or disease. Also, since such research requires large sample sizes, the inclusion of blood tests would make this approach feasible. Strategies recommended for the improvement of public health responses included the identification of methods for rapid notification of the risks for waterborne cryptosporidiosis to agencies, advocacy groups and the public.

The workshop participants emphasized that boiled water advisories are not essential in the absence of supporting epidemiological information suggesting increases in diarrheal disease in the community. On the subject of cryptosporidiosis in immunocompromised individuals, the workshop concluded that immunocompromised individuals are no more likely than immunocompetent individuals to acquire cryptosporidiosis in an outbreak. However, AIDS patients, patients receiving treatment for cancer, recipients of organ or bone marrow transplants and individuals who have congenital immunodeficiencies are at greater risk than immunocompetent individuals for developing severe, life-threatening cryptosporidiosis if they become infected.

Additional information on the epidemiologic aspects of cryptosporidiosis in humans is provided in the review of Casemore (1990). The distribution of *Cryptosporidium* in humans from several countries is broken down by age group, non-human reservoirs, and routes of transmission. This study found that when testing was performed for suspected infections, 60% of the positive findings occurred in children, and 30% in adults less than 45 years old. The disease occurred in single sporadic cases, small cluster cases, and short clinical series. *Cryptosporidium* is the third

or fourth most commonly identified pathogen in the world, and the reported rates are higher in underdeveloped countries, especially in children. Seasonal and temporal trends vary from country to country and occurrence may indirectly reflect rainfall and farming events such as lambing. Domestic animals such as calves and lambs in the U.K. are common zoonotic reservoirs involved in occupational exposure, indirect zoonotic transmission, and contamination of food (e.g., sausages, offal, and raw milk). Animals also contribute to environmental contamination in sources such as watersheds, food crops, and swimming pools. For example, 70 cases of cryptosporidiosis in the U.K. resulted from exposure to a single swimming pool with water contaminated by animal excreta. Pets, however, have not often been implicated as a source of infection and are not considered a major risk factor for acquisition of cryptosporidiosis (Glaser, 1998).

Person-to-person transmission of cryptosporidiosis may be associated with nosocomial (hospital-acquired patient-to-patient) infections, sexual transmission, and traveler's diarrhea (Casemore, 1990). Transmission is affected by ethnic and dietary differences (e.g., Muslims exhibit a lower prevalence than many other ethnic groups). Casemore observed that the severity of disease from infection is greatest among children under 5 years of age and among immunocompromised patients (e.g., AIDS or cancer patients), and that the impact is greatest in third world countries.

C. Treatment: Clinical Laboratory Findings and Therapeutic Management

Cryptosporidiosis is self-limiting in most immunocompetent patients and also in many immunocompromised patients. Recommended management of *Cryptosporidium* infected

patients includes careful monitoring of hydration and electrolyte balance with oral or intravenous hydration and nutrition as necessary. Patients with cryptosporidiosis-induced renal failure, liver disease, etc. may require treatment with a macrolide antibiotic (Griffiths, 1998). Antimotility agents (i.e. opiates or somatostatin and its analogues) may be helpful to prevent dehydration. Patients co-infected with HIV should continue or begin anti-retroviral therapy to suppress viral replication and boost CD4 cell counts. Patients currently undergoing chemotherapy or immunosuppressive therapy should be removed from treatment (Griffiths, 1998).

Since the publication of the 1994 *Cryptosporidium* Criteria Document, several new treatment strategies have been pursued. However, the search for a consistently effective treatment has fallen short. A comprehensive review of these efforts was published by Blagburn and Soave (1997) and O'Donoghue (1995). The use of spiramycin, a macrolide antibiotic, was described in the 1994 *Cryptosporidium* Criteria Document as showing limited success in the treatment of cryptosporidiosis. Other macrolides have been evaluated including clarithromycin (Jordan 1996), erythromycin (Connelly *et al.*, 1988), and azithromycin (Vargas *et al.*, 1993; DuPont *et al.*, 1996; Hicks *et al.*, 1996). Spiramycin and erythromycin have shown unacceptable toxicity, but azithromycin and clarithromycin have shown promise without the degree of side effects described for the earlier macrolides.

Favennec *et al.* (1994) tested anti-cryptosporidial activity of parmomycin and singefungin using a direct immunofluorescent assay in CaCo-2 cell cultures. Paromomycin has been successfully used for *in vivo* treatment of cryptosporidiosis although toxicity may be a problem (White *et al.*,

1995). Singefungin showed *in vitro* activity but requires further evaluation in humans (Favennec *et al.*, 1994). Ionophores with anti-coccidiocidal properties ve also been evaluated for treatment of cryptosporidiosis. The most promising, maduramycin and alboroxin, have shown 96% and 71% decreases of oocysts in immunodeficient mice respectively (Mead *et al.*, 1995). Bismuth subsalicylate (BSS), which has shown positive activity with a number of other diarrheal diseases, was evaluated for treatment and prophylaxis in challenged and infected immunodeficient mice (Greenfield *et al.*, 1996). Results were encouraging and further study with human patients was warranted. The use of hyperimmune bovine colostrum is thoroughly described by Crabb (1998) and shows potential for safe and effective treatment. However, considerable variation in efficacy has been noted (O'Donoghue, 1995). In summary, although there have been new discoveries of potentially effective therapeutic agents for humans, further research is required.

D. Mechanism of Action

The 1994 *Cryptosporidium* Criteria Document reports that the pathogenic mechanisms of *Cryptosporidium* are not known. While the pathogenesis remains unclear, more recent work has helped elucidate the process. *Cryptosporidium* sporozoites and merozoites invade the absorptive cells covering the small intestinal villi, damaging and eventually killing the enterocytes. Forney *et al.* (1996) suggested proteolytic activity is involved in the infectivity of *C. parvum* based on the interaction between human alpha-l-antitrypsin (ATT) and parasite subcellular components. This suggests the use of serine protease inhibitors may be useful as a therapeutic strategy. Riggs *et al.* (1996) identified antigens that may have a critical role in sporozoite infectivity and

therefore, may be suitable molecular targets for passive or active immunization against cryptosporidiosis.

Diarrhea occurs when intestinal absorption is impaired or secretion is increased. When killed enterocytes are extruded from the intestinal epithelium, crypt cells are signaled to repair the damage. Additionally, there is infiltration of prostaglandin (PGE) secreting inflammatory cells. Both crypt cells and PGE are known to stimulate chloride ion secretion; in addition, PGE inhibits NaCl absorption (Clark and Sears, 1996). This disruption in the absorption/secretion balance can lead to diarrhea. Clinical studies in *C. parvum* infected piglets (Argenzio *et al.*, 1993) have suggested that *Cryptosporidium*-induced diarrhea is of a secretory nature. However, Kelly *et al.* (1996) conducted perfusion studies to measure water and electrolyte transport *in vivo* in five HIV-cryptosporidiosis patients and nine healthy volunteers. There were no differences in net water, sodium, and chloride movement in the jejunum of the two groups. Neither was there evidence demonstrating that cryptosporidial diarrhea was due to a secretory state in the proximal small intestine. Other studies have suggested that *Cryptosporidium*-induced diarrhea may be caused by a toxin (Guarino *et al.*, 1994; Guarino *et al.*, 1995). A thorough review of cryptosporidial pathogenesis can be found in Clark and Sears (1996).

E. Immunity

The importance of cellular immunity in resolving *Cryptosporidium* infection is highlighted by the contrasting ability of immunocompetent and immunocompromised individuals to resolve infections. While depletion of CD8 cells (Ungar, 1990), NK cells (Rasmussen and Healy, 1992),

tumor necrosis factor (McDonald *et al.*, 1992), mast cells (Harp and Moon, 1991) or interleukin-2 (Ungar *et al.*, 1991) did not show enhanced infection in mice, removal of CD4 cells and/or gamma interferon caused severe chronic infections (Ungar *et al.*, 1991). Additionally, *Cryptosporidium*-infected immunodeficient mice reconstituted with spleen or lymph node cells from immunocompetent Balb/c mice, were able to recover from infection, but upon depletion of the CD4 T cells from the donor, the curative effects were abrogated (Kuhls *et al.*, 1996). In humans, HIV infected patients with CD4 counts of 180 cells/mm^3 cleared the infection in 4 weeks while of those with lower counts, 87% developed chronic disease (Flanigan *et al.*, 1992). Little is known about the gut mucosal response to the parasite. Wyatt *et al.* (1996) used a bovine animal model to examine mucosal immunity during cryptosporidiosis, and showed that ileal intraepithelial T lymphocytes are activated coincident with enteric disease. This suggests the importance of cell-mediated activity during *Cryptosporidium* infection.

Specific IgG, IgM, IgA, and IgE antibodies have been detected in patients with confirmed *Cryptosporidium* infection (Ungar *et al.*, 1986; Casemore, 1987; Laxer *et al.*, 1990; Kassa *et al.*, 1991). The presence of local and secretory antibodies has also been confirmed (Laxer *et al.*, 1990); however, the role of these antibodies in combating infection is unclear (O'Donoghue, 1995). Kapel *et al.* (1993) used a time-resolved immunofluorometric assay to determine the presence of *Cryptosporidium* antibodies in 12 HIV-*Cryptosporidium*-infected patients. These patients displayed marked elevation in anti-*Cryptosporidium* IgA and IgM antibody titers. These high antibody titers were not correlated with the gravity of infection in terms of oocyst shedding. Also, there was no evidence of protection even though there was a mucosal immune response.

Studies comparing *C. parvum* infections in B cell-depleted mice showed that infections were similar to those in normal mice (Taghi-Kilani *et al.*, 1990).

There is evidence for protective immunity to cryptosporidial infection. Repeat infections in dairy cattle workers occur but are generally much milder than the first infection (Reese et al 1982). Permanent residents in areas where cryptospordiosis is common often acquire mild or asymptomatic infections, however, visitors may become very ill. (Current, 1994) . Okhuysen *et al.* (1998) reports on the rechallenge of human volunteers previously infected with *Cryptosporidium*. Nineteen healthy, immunocompetent adults were challenged with 500 oocysts one year post primary infection. Fewer subjects shed oocysts after the second exposure (16% vs. 63%). Although the rates of diarrhea were similar, the clinical severity determined by the number of unformed stools passed, was less. The number of IgG and IgA seroconversions increased but the antibody response did not correlate to the presence or absence of infection.

F. Chronic Conditions

Duration of illness in cryptosporidiosis patients is influenced primarily by the immune response of the individual, with most immunocompetent individuals overcoming the acute enteritis stage within two weeks. Immunocompromised individuals generally present with chronic enteritis which may last as long as the immune impairment. Immunocompromised populations include patients undergoing chemotherapy for treatment of neoplasms, persons undergoing immune suppression treatment to prevent rejection of skin or organ transplants, malnourished individuals, patients who present with concurrent infectious diseases such as measles, the elderly, and AIDS

patients. Chronic illness may manifest itself as a series of intermittent episodes or may be persistent. A functional threshold has been established using CD4+ cells to define the probability that infection will resolve; patients presenting with CD4+ counts exceeding 200/iL can generally expect to clear the infection, while those with CD4+ counts falling below this level may suffer chronic infection (Fayer, 1997).

G. Summary

The primary symptom of cryptosporidiosis in humans is fulminant watery diarrhea. The limited data available from human volunteer feeding studies indicate a mean ID_{50} of 132 oocysts. Recent research suggests that the pathological response to *Cryptosporidium* is initiated when the sporozoites and merozoites invade and kill the intestinal epithelial cells (enterocytes). The enterocytes are extruded from the intestinal epithelium signaling epithelial repair and infiltration of inflammatory cells. The host responds with the production of antibodies as well as intraepitheleal T lymphocytes. The infection may cause malabsorptive and secretory diarrhea. Management of infected patients includes maintenance of fluid and electrolyte balance. Patients with unresolved infection may be treated with macrolides and anti-motility agents. A previous *Cryptosporidium* infection does not confer resistance to reinfection although reinfection will result in fewer episodes of diarrhea. A workshop panel focusing on the application of epidemiologic information on *Cryptosporidium* recommended making surveillance information available to the appropriate federal agencies, HMOs, hospitals, and others who play roles in maintaining the public health. Panel recommendations also included performing cohort analysis of outbreaks using information such as blood tests in populations where exposure to

Cryptosporidium is likely. In addition, workshop participants suggested strategies to improve public health, which included identifying methods for informing agencies, advocacy groups, and the public risks for waterborne *Cryptosporidium* transmission, and providing the public with information on dealing with a known or suspected contamination of drinking water source.

VI. Risk Assessment

The International Life Sciences Institute (ILSI) Risk Science Institute (RSI) Pathogen Assessment Working Group (1996) defined pathogen risk assessment as a process that evaluates the likelihood of adverse human health effects following exposure to pathogenic microorganisms in a medium such as water. Until recently, most formal risk assessments on pathogenic microorganisms such as *Giardia* and *Cryptosporidium* have utilized a conceptual framework that was developed to assess risks due to chemical exposures; however, it is notable that the framework for assessing chemical exposures does not account for a number of microbial considerations including pathogen-host interactions, secondary spread of microorganisms, short- and long-term immunity, the carrier state, host animal reservoirs, animal-to-human transmission, human-to-human transmission, and conditions that lead to propagation/multiplication of microorganisms. Although significant data gaps exist in the complete characterization of the pathogenesis of *Cryptosporidium*, risk assessment approaches will enable health officials to communicate with water utilities, interpret water quality surveys, and define the adequacy of treatment at acceptable public health risks (Rose *et al.*, 1997).

A. Experimental Human Data

The 1994 *Cryptosporidium* Criteria Document referred to human volunteer studies by DuPont *et al.* (1995) that were in progress at the time of manuscript preparation. The aim of the study was to determine the infectivity of *C. parvum* in healthy adults in order to predict the likelihood of enteric infection following exposure to contaminated drinking water. The results of this study have since been published and are presented in Table 4 of Section V-A. Among 29 subjects who were provided 30 or more oocysts, 62 percent became infected, with a range of 20% (30 oocysts ingested) to 100% (>1000 oocysts ingested). Illness lasted 58 to 87 hours with four to eleven loose stools produced per day, suggesting that human-to-human transmission of *C. parvum* is more likely to occur 2.5 to 3.5 days following infection in the primary case. Linear regression of the dose-response data indicated a human ID_{50} of 132 oocysts. The research team concluded that a 'low' dose of *C. parvum* oocysts was sufficient to cause infection in healthy adults with no serologic evidence of past infection by this parasite.

Follow-up analysis of the 18 individuals who presented with cryptosporidiosis infections indicated appreciable variation in the numbers of oocysts excreted and in the duration of excretion (Chappell *et al.*, 1996). Only 1 of the 7 volunteers who exhibited diarrhea had oocysts in every stool during the shedding period, and oocyst numbers in consecutive stool samples collected from individual volunteers varied as much as 30-fold. Hence, production of oocysts during infection may be intermittent, which may help to explain the observation that fewer than 50% of the individuals acquiring illness during waterborne outbreaks produce *Cryptosporidium*-positive stool samples when only one stool is examined.

B. Experimental Animal Data

A number of dose-response studies using monkeys, gnotobiotic lambs and several strains of mice are presented in the 1994 *Cryptosporidium* Criteria Document. Casemore (1990) reported a 2- to 5-day incubation period for *C. parvum* and an excretion period of about 8 to 14 days in animals (species not identified). DuPont *et al.* (1995) reported that the ID_{50} for the Iowa strain of *C. parvum* oocysts necessary to infect the neonatal mouse was 60, which is approximately half of the ID_{50} required to produce infection in humans (132 oocysts). The relative similarity among infectious doses in mice and humans may suggest that the mouse model is potentially useful in defining certain human risks associated with cryptosporidiosis.

C. Environmental Factors

1. Prevalence in Surface Waters

Cryptosporidium oocysts are more likely to occur in surface waters than in ground water, as described in Section III of this document and in the 1994 *Cryptosporidium* Criteria document. Since the majority of source waters used for production of drinking water are surface supplies, and because these waters are more vulnerable to direct contamination from sewage discharges and runoff, the presumption has been that *Cryptosporidium* will likely be more common in these supplies. Wallis *et al.* (1996) found *Cryptosporidium* oocysts in 6.1% of raw sewage samples, 4.5% of raw water samples, and 3.5% of treated water samples in Canada. Analyses of raw sewage samples indicate that *Cryptosporidium* was present in more than 50% of samples where one or more liters was examined (Bukhari *et al*, 1997; Zuckerman *et al.*, 1997). Ong *et al.* (1996 a and b) studied the source of parasite contamination in different watersheds to assess the potential impact upon drinking water sources, and found that water from rivers flowing through

cattle pastures in British Columbia exhibited higher *Cryptosporidium* counts than did water from a protected watershed. Lisle and Rose (1995) reviewed 25 monitoring studies and report *Cryptosporidium* in as much as 87.1% of the source waters, with levels of oocysts as high as 4.7 per liter. LeChevallier *et al.* (1995) observed oocysts in 60.2% of surface waters tested in North America. Crockett and Haas (1995) investigated three water treatment plants located in a major metropolitan area where watershed monitoring was conducted over one year. They found that creeks which empty into a river were a primary source of parasites in the urban river-derived water. Each creek's parasite density was greatly influenced by suburban wastewater discharges, although the authors did not rule out other sources which might have influenced the microbial density in the river. The implications of each of these studies should be tempered with the knowledge that erratic and insensitive oocyst detection methods may contribute to significant underestimation of the true level of oocyst contamination in surface waters.

2. Oocyst Survival

The resistance of oocysts to inactivation is an important factor in determining the extent to which humans or reservoir/host animals can become infected; however, most detection methods for *Cryptosporidium* can not distinguish between viable and nonviable oocysts. Furthermore, some detection methods may render oocysts nonviable due to chemical antagonism during sample processing. Additionally, the following practices must be characterized more completely in order to evaluate the risk of contracting cryptosporidiosis: sewage discharges, watershed protection, agricultural practices, wildlife management, strain specificity (animal or human) and oocyst survival under various environmental conditions. The survival of oocysts under various

environmental pressures has been evaluated by several groups and is described in Section III-G "Environmental Factors". The majority of these survival studies have relied upon animal infectivity or *in vitro* excystation to assess changes in oocyst viability in natural waters. A complete discussion on methods for the assessment of oocyst viability is also provided in Section VII.A.2 – "Detection of *Cryptosporidium* in Water".

3. *Cryptosporidium* in Drinking Water

Identification of the specific pathogen and route of infection represent early steps in the risk assessment process. The primary route of human infection by *C. parvum* involves ingestion of contaminated drinking water and food (Casemore, 1990); other routes of transmission are described in Section II-D. One of the difficulties in conducting risk assessment of *Cryptosporidium* lays in the uncertainty associated with the level of infectious oocysts in drinking water supplies. There are also viability, infectivity and specific epithet issues. Surveys such as those described in Section III-B have indicated the numbers of oocysts that may occur in drinking water; however, the impact of these oocyst levels may be underestimated given the number of gastrointestinal illnesses that occur each year whose etiology is undetermined.

No cases of cryptosporidiosis were reported in the waterborne disease outbreak survey published by the CDC from 1989 to 1990, however the large number of cases of acute gastrointestinal illness (AGI) of unknown etiology may have included illness caused by *Cryptosporidia*. Of the total number of AGI cases reported in 1989 and 1990, 56% (2,402 of 4,288) were unexplained (Herwaldt *et al.*, 1991). In the U.S. surveillance report from 1991 to 1992 (Moore *et al.*, 1993),

approximately 20% of the total enteric illness cases were caused by *Cryptosporidia*. Cases of AGI represented 76.5% of the total enteric illnesses reported from 1991 to 1992, many of which were likely caused by *Cryptosporidia*. These observations suggest that efforts to identify *Cryptosporidium* as the etiological agent during outbreaks frequently fail.

D. Epidemiologic Considerations

1. Population at Risk and Survey Methods

The USEPA estimated in 1993 that approximately 155 million people may be exposed to *Cryptosporidium* in contaminated water every year, however, the estimated population at-risk cannot be reconciled with the reported numbers of infected individuals even when correction factors for asymptomatic infections and underestimated environmental levels are included (1994 *Cryptosporidium* Criteria Document). Factors contributing to the disparity between the environmental occurrence data and the clinical data are outlined in the 1994 document; additional factors are described in Section V-B of this document. Hence, it is difficult to generate valid figures to describe the risk of acquiring cryptosporidiosis.

In the United States, the incidence of cryptosporidiosis in the U.S. can be assessed through surveillance reports and documentation of outbreaks that appear in the published literature. The CDC currently maintains an active surveillance system for cryptosporidiosis aimed at collecting information on both outbreak and non-outbreak related cases. Cases are reported using standard forms which originate with state and local health departments, but the agency also receives updates from federal agencies, and occasionally from private physicians. While cryptosporidiosis is not a reportable disease in all states (CDC, 1994), it is designated as

notifiable at the national level as of January 1, 1997.

A number of reports describe the severe effects of cryptosporidiosis in children (Molbak, 1994), particularly malnourished infants, although it is generally difficult to determine if malnourished children are at higher risk of chronic cryptosporidiosis due to immune suppression, or if cryptosporidiosis is an independent risk factor for becoming malnourished (Griffiths, 1998). Reports from the U.K. show the occurrence of cases to be highest among children less than 5 years old (Atherton *et al.*, 1995). Other groups at risk for cryptosporidiosis are secondary contacts, farm workers (Lengerich *et al.*, 1993), immune-suppressed individuals, those living in institutional settings such as group homes and orphanages (Heald and Bartlett, 1994), and international travelers who visit regions where cryptosporidiosis is endemic. There is little evidence that risks differ between genders (Meinhardt *et al.*, 1996).

E. Risk Assessment Models

Since *Cryptosporidium* monitoring does not presently give a true picture of the number of infectious particles and the efficacy of removal of oocysts from treated drinking water, risk calculations involve many uncertainties. In order to develop risk estimates for specific pathogens such as *Cryptosporidium*, reliable dose-response data are required. The human dose-response data currently available is limited to the studies of DuPont *et al.* (1995) and Chappell *et al.* (1996); however, an exponential dose response model has been developed based upon this dataset and a number of assumptions governing the Milwaukee epidemic of cryptosporidiosis (Haas, 1994). This model describes the probability of infection given exposure (P_I) as follows:

Equation 1. $P_I=1-e^{-rN}$

The values of r and N represent the fraction of ingested oocysts which must survive to establish infection, and the daily exposure, respectively. A value of r specific for *Cryptosporidium* has been derived (r=0.0047). Oocyst concentrations were derived for exposures ranging from 1 to 30 days and a P_I range of 0.14 to 0.52. Exposure values predicted according to this model ranged from a minimum of 0.16 oocysts per liter (P_I=.14) to a maximum value of 79 (P_I=0.52). *Cryptosporidium* concentrations in the Milwaukee water supply were derived by considering the numbers of oocysts present in ice produced during the epidemic and correcting for losses associated with poor analytic recovery efficiencies. According to the exponential model, *Cryptosporidium* exposure during the epidemic ranged from 0.6 to 1.3 oocysts per liter. Haas also applied the risk assessment model to consider data from previous water monitoring studies, and has calculated that the annual risk of contracting cryptosporidiosis in the United States may range from 1 in 100,000 to 4 in 1,000.

Perz *et al.* (1998) applied a risk assessment approach to examine the role of tap water in waterborne cryptosporidiosis. This model was based upon the assumption that clinical infection results from exposure to a single oocyst, and utilized a theoretical *C. parvum* density in drinking water of 1 oocyst per 1,000 liters. Uncertainties in the model were analyzed by considering ranges and distributions among the input variables. The number of annual *Cryptosporidium* infections (I_j) was estimated according to the following relationship:

$$I_j = C \cdot POP_j \cdot Q_j \cdot r_j$$

Where C = concentration of *C. parvum* per liter of water
j = population subgroup (categorized by age and AIDS status)
POP_j = number of persons in the exposed subgroup

Q = annual water intake in liters per year
r = single organism infectivity (infection/organism/person)

The model was applied to derive the median annual risk of infection among immunocompetent individuals (1 in 1,000 probability using the assumed exposure level of 1 oocyst per 1,000 liters). The dominant parameter contributing to uncertainties in the risk assessment was oocyst concentration, suggesting that improvements in *Cryptosporidium* monitoring techniques in drinking water will facilitate future risk assessment efforts.

The ILSI workgroup (1996) has developed guidance for an infectivity model that may also be useful if adequate detection methods are available. Since exposure analysis for humans and animals is an essential step in risk assessment, developing animal systems that can be used to estimate infectivity will be essential for modeling because detection of oocysts without knowledge about their infectivity is inadequate. Varga *et al.* (1995) reported on a model using *C. baileyi* infections in chickens. The authors developed a quantitative method to assess oocyst shedding which was based on the rather slow sedimentation of oocysts. A threshold of sensitivity of between 5,000 and 10,000 oocysts per gram of feces was reported for the technique. Triplicate assays over a wide range of oocyst concentrations (i.e., 2,500 to 1.25×10^6) were in good agreement. Considering the level of oocysts excreted by infected humans, this model system appears to have adequate sensitivity for demonstrating infection. This model's sensitivity threshold may be adequate for assessing stool samples of infected individuals, but is inadequate for monitoring oocysts in water samples for potential infectivity; hence, further development of sensitive infectivity models is needed.

F. Federal Regulations

Cryptosporidium as a primary contaminant is currently not specifically regulated by the federal government. However, since the 1994 *Cryptosporidium* Criteria Document was published there has been a substantial amount of federal regulatory activity associated with *Cryptosporidium* in drinking water primarily prompted by the 1996 Amendments to the Safe Drinking Water Act. USEPA has initiated numerous stakeholder meetings and has several new and pending rules that are being driven by the threat of *Cryptosporidium* water-borne disease. Lykins and Clark (1994), Berger (1996) and Pontius (1998) have published exhaustive review articles summarizing these activities. The most significant promulgated and pending rules are the Information Collection Rule and the Enhanced Surface Water Treatment Rule, respectively.

On May 14, 1996 the USEPA promulgated the Information Collection Rule (USEPA, 1996a). The rule requires those water utilities serving more than 100,000 people to test source water and finished water for an eighteen-month period (the process began in July 1997). The monthly testing includes a variety of analytes including coliforms, turbidity, and *Cryptosporidium*, etc. The rule is primarily a research effort and the USEPA intends to use the information for the development of future rules. The USEPA outlined its intended approach for regulation in the November 3, 1997 ***Federal Register*** (USEPA, 1997). In this document, USEPA states that a final Interim Enhanced Surface Water Treatment Rule is scheduled for November, 1998. The rule will set a maximum contaminant level goal (MCLG) of zero for *Cryptosporidium*. While *Cryptosporidium* is primarily considered a threat to surface water supplies, recent research with vertical wells, springs, and infiltration galleries suggest that groundwater sources may also

harbor the parasite (Hancock *et al.*, 1998). The USEPA is currently convening stakeholder meetings preliminary to expected promulgation of the Groundwater Disinfection Rule in early 1999.

G. Summary

Environmental risk assessments have historically relied upon a conceptual framework based upon exposure to chemical pollutants and are generally considered inadequate for pathogen risk assessment. Although most human populations are assumed to be at risk for cryptosporidiosis at least to some degree, it has been difficult to collect accurate figures describing the prevalence of infection in humans due to limitations in public health reporting systems and due to incomplete characterization of oocyst speciation and survival under various environmental conditions. Risk assessment for waterborne cryptosporidiosis has also been limited due to the absence of dose-response data from volunteer challenge. Data from animal infectivity studies suggest that infectious doses may be lower (ID_{50} of [illegible] oocysts). Risk models have been developed to assess the probability of cryptosporidiosis infection based upon assumptions governing the levels of infectious oocysts in drinking water and upon the data generated from volunteer challenge studies. The estimated annual risk of waterborne cryptosporidiosis based upon these models ranges from 1 in 1,000 to 1 in 100,000.

VII. Analysis and Treatment

A. Analysis of Water

The Information Collection Rule modified American Society of Testing and Materials method (ASTM ICR), described in the 1994 *Cryptosporidium* Criteria Document, remains the most commonly used method for monitoring *Cryptosporidium* in water. The method, which detects both *Cryptosporidium* and *Giardia*, is tedious and requires high levels of technical expertise. While reproducible and sensitive for *Giardia* detection, the method is not reproducible between laboratories, and suffers from low sensitivity for *Cryptosporidium* detection (Clancy *et al.*, 1994; USEPA, 1996b).

The accuracy and reproducibility of method development and comparison studies was questioned by Klonicki *et al.* (1997). The authors noted that vital information regarding oocyst source, purification method, age, storage conditions, and enumeration method was inadequately cited in most publications. Further studies compared three enumeration methods; hemacytometer, membrane, and well slide, and showed statistically significant differences among the methods. The authors demonstrated the effect of counting method variability on recovery values and stressed the importance of standardizing method comparisons and providing adequate information in publications to allow valid comparison studies.

LeChevallier *et al.* (1995) investigated numerous methodological variations in the collection, elution, and concentration portions of the assay to determine their influence on recovery of *Cryptosporidium* in seeded tap and Mississippi River water samples. The authors tested multiple filter types, centrifugation speeds, density gradient modifications, elution buffer pHs,

and centrifuge bottle types. Although they identified several sources of oocyst loss, their modifications resulted in variable recovery (7-129%).

Before detection data can be applied in a meaningful way, it must be adjusted to reflect the limits of the methodology used to collect it. For example, if the method can detect 1,000 oocysts in 100 L of water and a sample contains only 50 oocysts, a result showing 0 oocysts could be obtained. The results, therefore, must be reported to say less than 1,000 oocysts are present. Harris (1995) developed a method detection limit for IFA analysis of *Cryptosporidium* and *Giardia*. The $MDL_{0.95}$ in this study is defined as the minimum concentration that the procedure can analyze with 95% confidence that the organism will be detected.

Each water type presents unique problems in collection, concentration, isolation, and staining techniques. Organic and inorganic particulates present in the water can clog filters or interfere with other portions of the analysis such as clarification or antibody staining. Additionally, the ASTM ICR immunofluorescent staining methods do not give information regarding viability and infectivity and speciation which is essential to assessing public health threat. The reader is advised to review Table 3.19 in Frey, *et al.* (1998) for a complete synthesis of research status of *Cryptosporidium* detection methods. Efforts to develop improved *Cryptosporidium* collection, concentration, and detection methods are described below.

1. Collection of *Cryptosporidium* from Water

Filtration based concentration methods

The 1994 *Cryptosporidium* Criteria Document describes the most common collection method using polypropylene wound yarn filter with 1 μm porosity. This collection method remains the most popular and can be used for large volume samples with varying turbidity. LeChevallier *et al.* (1995) tested ten cartridge filters varying in composition (polypropylene, nylon, rayon, and cotton) and porosity (0.5 and 1.0 μm) for removal of *Cryptosporidium* and *Giardia*-sized particles. Although retention of 3 and 7 μm particles was greater using filters with a 0.5 μm porosity, they tended to clog, limiting the amount of water that could be filtered. The use of cotton, nylon, and rayon filters led to the most efficient removal of *Cryptosporidium* and *Giardia*-sized particles. To further minimize losses during filtration, the filter housing was matched with the filter and a screw press was used to wring the filters. Concentration of the eluate was best performed at centrifuge speeds of 6,700 to 10,000 x g.

Also described in the 1994 *Cryptosporidium* Criteria Document is the use of cellulose acetate membrane (CAM) filters. Nieminski *et al.* (1995) compared recovery rates of a method using CAM filters to the ASTM ICR method using wound yarn filters. *Cryptosporidium* and *Giardia* were spiked into environmental water samples varying in quality and turbidity prior to filtration by either method. Cyst and oocyst recoveries decreased with increasing water turbidity regardless of the filter type. Overall, the cellulose acetate method gave higher recoveries, however, because the parasites were stained on polycarbonate filters, microscopic confirmation was not possible. Therefore, the authors recommended the use of the ASTM ICR method for environmental sample analysis and the cellulose acetate method for spiking studies. Adlom and

Chagla (1995) modified the CAM method by including an acid dissolution step following filtration. This modification resulted in a 70.5% recovery of oocysts spiked into 3 liters of treated municipal water. Graczyk *et al.* (1997) used cellulose acetate filters, followed by filter dissolution and ASTM ICR method processing to test recovery of *Cryptosporidium* from spiked drinking water. The overall mean recovery rate was reported at 77.7%. Further studies by Graczyk *et al.* (1997) indicate the acetone dissolution step does not compromise viability or infectivity.

Envirocheck™ capsules contain a pleated polysulphone membrane with a 1μm porosity. This 6 cm diameter x 21 cm long capsule has a surface area of 1300 cm^2. Clancy (1997) compared throughput and recovery rates of this capsule filter with those of polycarbonate membrane filters, vortex flow filtration, and cellulose acetate membrane filters which were dissolved post filtration. All four filters were challenged with 10 liters of municipal raw and finished waters. Cellulose acetate membranes and polycarbonate membranes blocked at 8 and 2.5 liters respectively, at a raw nephlometric turbidity unit (NTU) of 5. The polymer vortex flow and Envirocheck™ capsule filters were able to process the entire 10 liters of raw water and gave recovery rates of 11-57% and 8-78% respectively. In finished waters from 5 utilities, the vortex flow recovered 18-69% of the seeded oocysts while the capsule filter recovered 45-117%. The researchers concluded the capsule filter performed best with the various water matrix conditions tested. Other membrane filters composed of glass fiber have been evaluated and shown to be of poor integrity (Whitmore and Carrington, 1993).

Centrifugation based concentration methods

Vortex flow filtration (VFF) is centrifugation-based filtration method in which a water sample is passed through a cylindrical membrane filter rotating at high speed within a second cylinder. The rotation sets up forces that scrub the membrane surface and prevent blockage by particulates. The liquid phase (permeate) crosses the filter while the particulate containing phase (retentate) is continuously recirculated and concentrated. Whitmore and Carrington (1993) evaluated the ability of a VFF apparatus with a 0.45μm polysulphone membrane cartridge filter, followed by clarification step using density gradient centrifugation, to concentrate *Cryptosporidium* from spiked bore hole or river water samples. The VFF device recovered between 30 and 40% of the seeded oocysts. Fricker (1997) reported greater than 60% recovery using VFF in seeded river water samples.

Whitmore and Carrington (1993) evaluated cross-flow filtration for recovery of oocysts in clean water. This apparatus pumps water across a set of alumina filters in a parallel series. In this study, the retentate, typically 150-200 ml was collected, centrifuged, resuspended in phosphate buffered saline (PBS), and counted using a hemacytometer under Nomarski differential interference contrast (DIC) illumination. The authors note this method is rapid and recovered 70% of the seeded oocysts in small volume samples. Studies using larger volume samples recovered 40%. The authors suggest more effective cleaning or replacement of the filters between runs may obtain higher recovery rates. The device produces small volumes of retentate facilitating further concentration, is compact, and can be sterilized.

Researchers at Marshfield Clinic in Marshfield WI have developed a continuous centrifugation method to concentrate parasites from water (Borchardt and Spencer, 1996). This method uses a blood cell separator, which operates on the principal of channel-type centrifugation, to concentrate oocysts from water samples. Recoveries over a range of water turbidities, oocyst concentrations, and water volumes were reported to be 78-101% .

Concentration using flocculation

Calcium carbonate flocculation has also been used to concentrate *Cryptosporidium* in up to 10 liters of water. The method, developed by Vesey *et al.* (1993b), uses calcium chloride and sodium bicarbonate in a high pH solution to crystallize organic particles. The crystals are allowed to settle, the supernatant is discarded, and the calcium carbonate precipitate is dissolved with sulfamic acid. Vesey *et al.* (1993b) reported recoveries greater than 68% with this method. Subsequent analyses of this method by Shepherd and Wyn-Jones (1995 and 1996) were in agreement and reported calcium carbonate flocculation consistently gave higher recoveries when compared to cellulose acetate membrane and cartridge filters. However, calcium carbonate flocculation is not recommended for drinking water analysis when viability is important. Studies by Robertson *et al.* (1994) showed significant viability reduction, determined by vital dye staining (See Viability, VII.A.2) following 4 hour exposure to a pH of 10.

Clarification by density gradient centrifugation

Density gradient floatation methods are commonly used to clarify samples and concentrate oocysts prior to detection. Centrifuge speed and time, and density of the solution vary among

laboratories using this method. LeChevallier *et al.* (1995) reported a Percoll sucrose gradient with a specific gravity of 1.15 was 67% better than a gradient with a specific gravity of 1.0 for recovery and concentration of oocysts. A gradient with a specific gravity of 1.3 did not clarify the sample and interfered with microscopic analyses. This study also indicated that floatation selects for empty oocysts while live, intact oocysts sink to the bottom of the gradient rather than floating to the upper fraction (LeChevallier *et al.*, 1995). Shepherd and Wyn-Jones (1995) reported significant decreases in recovery (>50%) when sucrose flotation techniques were included in detection methods.

Flow cytometry

A flow cytometer is a laser-based instrument that analyses particles in a liquid suspension on a particle by particle basis. It can differentiate and physically separate (sort) particles based on their size, internal complexity, and fluorescence. Flow cytometry with cell sorting (FCCS) is used routinely in the UK and Australia for detection of *Cryptosporidium* and *Giardia* in environmental samples (Vesey, 1993a,1994). Briefly, a concentrated portion of the sample is incubated with a fluorochrome conjugated monoclonal antibody that binds a portion of the oocyst wall causing the organism to fluoresce. The instrument analyses the particles and electrically charges those selected by the operator based on a signal (eg. fluorescence). The charged particles are pulled out of the sample stream using oppositely charged electrical plates and deflected onto a microscope slide. The slides, free of the debris which can obscure oocysts on a membrane filter slide, can be read in 5 to 10 minutes as opposed to 90 - 120 minutes cited for membrane analysis. In studies published by Vesey *et al.* (1994), FCCS detected greater than

92% of the *Cryptosporidium* and *Giardia* in seeded river and reservoir samples. Additional work incorporating a calcium carbonate flocculation concentration step prior to FCCS reported 64.1% and 62.7% recovery of the oocysts seeded in filtered and raw waters, respectively (Veal, 1997). Studies by Hoffman *et al.* (1997), using a variety of environmental samples, reported that FCCS detected three times more *Cryptosporidium* positive samples than membrane IFA (94.1% vs. 35.3%, respectively) and an equal number of *Giardia* positives samples. This technique has been repeatedly shown superior to traditional membrane IFA analysis (Danielson, 1995; Compagnon, 1997). Researchers at Macquarie University in Australia, working with a flow cytometer manufacturer, have modified the traditional flow cytometer to optimize it for water quality analysis. This type of flow cytometer is not currently available in the US. The FCCS method provides increased sensitivity, and requires less time, expense, and experience than the ASTM IFA. Additionally, this method can, with no extra effort, simultaneously detect *Giardia*. Disadvantages of this method include the initial expense of the instrument ($150,000-200,000) and the level of flow cytometry expertise required with the non-optimized models.

Immunomagnetic separation

Immunomagnetic separation concentrates *Cryptosporidium* using magnetic beads coated with an anti-*Cryptosporidium* antibody. Following elution, the sample is incubated with magnetic beads that bind the oocysts. The solution is inserted into a magnetic particle concentrator that binds the magnetic bead-*Cryptosporidium* complex. After the supernatant is decanted, the beads are released from the magnet. Oocysts are dissociated from the magnetic particles using an acid wash, neutralized with base, and subjected to analysis. This method was evaluated for

Cryptosporidium by Robertson and Smith (1992); later efforts to develop a *Giardia* detection system were pursued by Bifulco and Schaeffer (1993) who reported an 82% recovery rate for *Giardia* in waters of varying turbidities. Campbell *et al.* (1997) spiked laboratory grade and turbid water samples with *Cryptosporidium* and concentrated them using either ASTM IFA, the UK Standing Committee of Analysts method (SCA), or IMS. Immunomagnetic separation gave the highest recoveries in either water type. Clean water recoveries using IMS were 97.4% while the ASTM IFA and SCA methods recovered 26.9 and 19.3% respectively. Turbid water recoveries were 65.8% using IMS, 5.4% with the ASTM IFA, and 11.7% with SCA. A five site evaluation comparing IMS to FCCS and a modified SCA reported the IMS method was superior to the FCCS and SCA methods in low turbidity waters. However, there was a direct relationship between turbidity and performance of the IMS method. Flow cytometry showed the greatest recoveries in higher turbidity waters (Campbell *et al.*, 1997). Fricker *et al.* (1997) evaluated the IMS procedure in water samples seeded with 100 oocysts. Recovery rates ranged from 71-120%.

Panning

Panning is a technique originally developed to isolate specific cell types from a mixed cell population. An antibody raised to the target is attached to a solid substrate and incubated with the target cell containing suspension. During the incubation period, the target cell or organism is bound by the antibody and the remaining cells or debris can be washed off. Direct and indirect panning methods were tested by Stone (1997) to concentrate *Cryptosporidium* oocysts during the clarification stage. The authors recovered 50% of the oocysts seeded into Hanks Balanced Salts Solution (HBSS) by direct panning and 20% of the seeded oocysts using indirect panning.

2. Detection of *Cryptosporidium* in Water

Indirect Immunofluorescence assay

Direct and indirect immunofluorescent antibody detection methods facilitate the visualization of oocysts which may be obscured by debris in environmental samples. The indirect immunofluorescence assay (IFA) (Fout *et al.*, 1996), described in the 1994 *Cryptosporidium* Criteria document, remains the most widely used detection method. The test, however, does not provide information regarding viability, infectivity, or species. The HydroFluor Combo staining reagents have been shown to cross react with various algal species (Rodgers *et al.*, 1996) and an experienced microscopist is essential for accurate and reliable examination.

Well slide staining

Well slide staining has been evaluated as an alternative to membrane IFA staining (Frederickson *et al.*, 1995). In a five site side by side comparison, *Cryptosporidium* recoveries using the well slide method were in excess of 50% higher than those obtained using traditional membrane. Additionally, the well staining procedure took 50% less time to perform. The authors suggested the physical forces of the vacuum used in membrane IFA staining may result in destruction of cysts and oocysts. Microscopic examination of the contents of the Hoefer tank accounted for a 78% loss of *Cryptosporidium* with 2% remaining intact. *Cryptosporidium* have been shown to be compressible (Li *et al.*, 1995) and may slip through filters with pore sizes smaller than their 4-6 µm diameter.

Enzyme immunoassays

Traditional enzyme immunoassays can provide rapid detection of oocysts with little tedium. While these assays have been used clinically, their use in environmental analysis is not common. Several papers have indicated the ability for their use with environmental samples. (Siddons, 1991;Chapman, 1990; Gracyzk *et al.*, 1996b). In one instance, Siddons (1991) reported a positive EIA detection of one oocyst. Chapman and Rush (1990) reported EIA sensitivity equal to microscopic examination in both environmental and human samples. de la Cruz and Sivagansen (1994) tested five *G. lamblia* EIA kits and two *C. parvum* EIA kits for detection of cysts and oocysts respectively in buffered saline and river water. The authors found some kits were capable of detecting <10 cysts or oocysts, however, results were variable with fixed and unfixed organisms. The authors also found that disruption of the parasites increased the kit's sensitivity. Each EIA kit's performance was influenced turbidity and some kits cross reacted with algae. Gracyzk *et al.* (1996b) compared the specificity of the Prospect T™ enzyme-linked immunosorbent assay (ELISA) to that of the HydroFluor Combo antibody used in the ASTM ICR method, and the Merifluor direct stool kit antibody. Their results showed the ELISA gave a positive reaction with only 6 of 25 non-*C. parvum* isolates tested, while the HydroFluor combo and Merifluor direct antibodies each cross reacted with 19.

Molecular methods: Polymerase Chain Reaction Assays

Several reports describe various applications of the polymerase chain reaction™ for the detection of *Cryptosporidium* in drinking water (Rochelle *et al.*, 1997 a and b; Johnson *et al.*, 1995b; Wagner-Wiening and Kimmig, 1995; Filkorn *et al.*, 1994; Johnson *et al.*, 1993). These methods

rely upon *in vitro* enzyme-mediated amplification of *Cryptosporidium*-specific nucleic acids in order to facilitate identification in water samples, which should in theory offer unmatched endpoint sensitivity and the possibility of distinguishing subtle differences among discrete strains of parasites. A number of techniques aimed at distinguishing viable and nonviable oocyst populations are also reported. These applications are summarized below; however, it is important to emphasize that due to the enzymatic nature of the PCR process, these assays may fail due to inhibition of enzyme activity caused by compounds commonly found in natural waters. Additionally, the majority of the documented studies have been limited to evaluations of seeded water samples rather than actual comparative field trials. Hence, the application of these techniques toward the detection of *Cryptosporidium* in environmental water samples should be considered developmental.

Johnson *et al.* (1995) reported a PCR protocol to detect *Cryptosporidium* in environmental samples, based upon oligonucleotide primers specific to a portion of the small 18S ribosomal RNA. Detection sensitivities of 1 to 10 oocysts were achieved in purified oocyst preparations, however, the detection sensitivity in seeded environmental samples was up to 1000-fold lower. Poor endpoint sensitivity was at least partially offset by concentration by flow cytometry or immunomagnetic capture and oligoprobe hybridization using a chemiluminescent technique. These methods were applied to confirm the presence of oocysts in water samples from the Milwaukee outbreak of cryptosporidiosis, and the results of the PCR assays were comparable to those observed when immunofluorescence methods were practiced.

Rochelle (1997b) evaluated four pairs of previously published primers aimed at the specific detection of *C. parvum*. Detection sensitivities ranged from 5 to 50 oocysts in seeded environmental samples when PCR was followed by oligoprobe hybridization, with some primer pairs offering species specificity while others were only genus-specific. Successful multiplex reactions aimed at the simultaneous detection of *G. lamblia* and *C. parvum* were evaluated and demonstrated the utility of PCR for the detection of waterborne parasites; however, no primer combinations were identified which exhibited the ideal combination of sensitivity, specificity and compatibility with multiplex reactions, and several primer sequences reported previously failed to amplify their targets.

Since PCR is capable of detecting the genetic material of both live and dead microorganisms, a number of studies have targeted unique sequences to distinguish viable from nonviable oocysts. Wagner-Wiening and Kimmig (1995) applied PCR to detect *Cryptosporidium* targeting a large DNA fragment specific to *C. parvum*. In order to differentiate between live and dead oocysts, this group reported the practice of applying an excystation protocol prior to PCR and targeting sporozoite DNA to ensure that amplified material was associated only with viable oocysts. Endpoint sensitivity as low as 100 sporozoites was observed, and was reduced to 10 sporozoites when nested PCR was practiced. Filkhorn *et al.* (1994) also evaluated RNA-based measurement of viable oocysts by practicing excystation prior to PCR. To preclude spurious contamination caused by the presence of DNA in nonviable parasites, DNAse enzyme was applied to digest free DNA, leaving only free RNA from viable oocysts.

Stinnear *et al.* (1996) describe a reverse transcription PCR (RT-PCR) detection method specific for *C. parvum* which can detect single viable oocysts based upon the assumption that only viable oocysts are metabolically active and will produce messenger RNA (mRNA). Since mRNA exhibits an extremely short half life in living cells (and perhaps even shorter outside of the protective environment of the oocyst wall), only the mRNA from viable oocysts will be captured and amplified.

PCR has also been used to track *Cryptosporidium* reductions during water treatment. Mayer and Palmer (1996) evaluated several methods to identify *Cryptosporidium* oocysts in sewage and to determine reductions during wastewater treatment. The nested PCR technique described was compared to a modified ASTM method to track reductions during treatment, and strong correlations were observed, with approximately 2 $\log_{10}$ *Cryptosporidium* reductions observed. The authors concluded that the PCR method was preferable due to a substantial reduction in sample collection and processing during analysis.

Molecular methods: Cell culture-Polymerase Chain Reaction

At least one integrated approach has been reported utilizing tissue culture, PCR and in situ PCR (IS-PCR) to assess seeded natural water concentrates for the presence of infectious oocysts (Rochelle, 1997a). This technique offers the possibility of screening out noninfectious oocysts since only the infectious oocysts will develop during the initial phase of amplification in human adenocarcinoma cells. Preliminary experiments suggest that IS-PCR may offer quantitative detection of infectious oocysts in natural water concentrates.

Molecular methods: Strand displacement amplification

A strand displacement assay for the detection of *Cryptosporidium* in natural waters samples has been reported to overcome the comparatively long cycle times associated with PCR (Blassak *et al.*, 1996). This method is based upon the selective replication of a single DNA strand while the other parental strand is displaced from the template and colorimetric detection of oocyst DNA under a microplate format. Low *Cryptosporidium* densities (~1) were detected in deionized water; reaction inhibition associated with seeded finished and raw water samples resulted in lower sensitivity.

Laser scanning microscopy

The ChemScan RDI is a laser scanning device linked to an epifluorescent microscope with a motorized stage. Sample analysis includes a filter staining procedure, followed by automated scanning with the ChemScan instrument. The instrument digitizes the location of fluorescent objects allowing for quick confirmation. This instrument has the advantage of being highly automated and more sensitive than FCCS and IMS methods in samples with colloidal clay (Reynolds *et al.*, 1997). The recovery rate and minimal detection limit were not specified nor was the oocyst spike concentration. Disadvantages of this method include the cost of the instrument ($200,000-300,000) and use of membrane filters which have been shown to contribute to oocyst loss (Frederickson *et al.*, 1995), and the inability to perform any light microscopy techniques for visualization of internal cytoplasm or sporozoites.

Miscellaneous methods

Campbell *et al.* (1993a) contrasted flow cytometry and a slow scan cooled charge couple device (CCD) for detection of *Cryptosporidium*. The authors discuss the advantages of the CCD, including its ability to simultaneously assess viability by DAPI staining, however, a comparison of results is not provided. The need for sophisticated and currently unavailable software is indicated.

Campbell *et al.* (1993b) evaluated enhanced chemiluminescence for detection of *Cryptosporidium* in 21 environmental samples previously assayed by microscopy. The oocysts were labeled with a FITC conjugated anti-*Cryptosporidium* antibody, followed by a biotin conjugated anti-FITC antibody and streptavidin-peroxidase. Statistical analysis revealed no difference between the enhanced chemiluminescence assay and microscopy. The authors report the chemiluminescence method is faster than the currently used microscopic method, however, visual confirmation was still necessary.

The electrorotation assay (ERA) relies on the principle that small particles can be induced to rotate in the presence of a rotating electric field. The rate of rotation is due to the field and surface charge of the particles. Jakubowski *et al.* (1996) described a proprietary electrorotation assay in which oocysts are attached to an antibody-coated magnetic bead and placed in a filter electrode assembly. The assembly is placed under a microscope, connected to an electric field generator, and the number of rotating oocysts are enumerated. This method may be able to differentiate viable and non viable oocysts based on differences in their rotation rates. Oocyst recovery rates have been reported from 30-95%, however, the efficiency of this method is

dependent on the type and characteristics of the water. A major disadvantage to ERA is that magnification can only be performed up to 400x due to the thickness of the ERA unit.

Method 1622

Recognizing the need for an improved *Cryptosporidium* detection method, the EPA initiated an effort to identify new and innovative technologies for protozoan monitoring and analysis. Following a comprehensive evaluation of existing and emerging technologies, the EPA Office of Water developed an initial draft of Method 1622 in December 1996 (EPA-821-R-97-023). This method continues to be modified and is currently being tested in several laboratories across the US.

A 10 L sample is shipped to the laboratory where it is filtered through a polysulfone capsule filter. Following filtration, the capsule is filled with eluting solution and shaken on a wrist action shaker for approximately 5 minutes. The capsule is drained and the elution procedure is repeated. The combined eluate is concentrated by centrifugation, reconstituted to 10 ml, and subject to IMS as described previously. The solution is stained with anti-*Cryptosporidium* antibodies using the well slide method described previously.

Several options are listed in the EPA Method 1622 draft document including concentration by vortex flow filtration or membrane disk filtration and an alternative immunomagnetic separation procedure. Additionally, a draft document outlining flow cytometry coupled with cell sorting as an alternative to the IMS and well slide analysis has been written.

Viability determinations

The public health significance of *Cryptosporidium* relates primarily to the ability of this parasite to initiate infections in humans and animals. Although the gold standard among infectivity assays remains the animal model, the high costs associated with assays using animals and ethical considerations precludes their routine use. Additionally, these methods do not offer adequate sensitivity for testing of environmental samples where the level of oocyst contamination may be on the order of 1 or 2 oocysts per liter; additionally, differences in pathogenesis among humans and animals have called into question the applicability of animals in providing an accurate reflection of cryptosporidiosis in humans. Nonetheless, innovative viability assessment methods for *Cryptosporidium* are inevitably compared to animal models, primarily using mice, and these methods are described below.

In vitro excystation (IVE) estimates infectivity by determining the number of potentially infectious oocysts by simulating the conditions of the mammalian gastrointestinal tract. Oocysts exposed to an acid pretreatment and bile salts at elevated temperatures will release progeny sporozoites (excyst), whereas metabolically inactive (nonviable) oocysts will fail to excyst. Excystation is tracked by direct microscopic visualization of treated samples and performing a comparison to the initial untreated population. Black *et al.* (1996) and Belosevic *et al.* (1997) indicate that IVE assay may significantly overestimate the true infectivity of oocysts treated with chemical antagonists when compared to results of animal infectivity studies. Although IVE assay is relatively time-consuming and is generally not applicable toward tracking reductions during water treatment (which may exceed several orders of magnitude), Vesey *et al.* (1997) has

reported an adaptation using flow cytometry to increase the sensitivity and throughput and has observed good agreement with the microscopic method.

The *Cryptosporidium* Criteria Document (1994) described the early work of Campbell which focused on vital dye staining. This group evaluated the application of dyes which are preferentially absorbed by viable oocysts and compared the technique with *in vitro* excystation, where strong correlations were observed. This work was confirmed by Bukhari (1995) who compared vital dye staining and IVE of oocysts concentrated using several different methods. Subsequent studies by Black *et al.* (1996) indicated that vital dye staining correlated well with IVE but had a tendency to overestimate infectivity of ozone-treated oocysts when compared to animal infectivity. Jenkins *et al.* (1997) confirmed that vital dye staining tends to overestimate infectivity relative to animal infectivity, but supported its use as an economical, user-friendly method that provides information on the effects of stresses on the surface of oocysts over time. Belosevic *et al.* (1997) evaluated 14 nucleic acid stains as indicators of *Cryptosporidium* viability and compared the staining to both animal infectivity and IVE assay. Both heat-treated and chemically inactivated oocysts were consistently stained with novel stains (SYTO-9, SYTO-59, and hexadium). This staining correlated well with animal infectivity but not IVE assay. The authors also developed an IFA-viability assay relying only on propidium iodide (PI) for viability assessment; however, extraneous factors such as aldehyde fixation may inhibit uptake of PI and falsely elevate the numbers of viable oocysts (Campbell *et al.*, 1993c).

Various infectivity assays have been described using tissue culture methods to assess the

ıfectivity of *C. parvum* (Upton *et al.*, 1994a; Upton *et al.*, 1994b; Upton *et al.*, 1995; Rochelle *t al.*, 1997a; Slifko *et al.*, 1997). These methods track the development of progressive *Cryptosporidium* infections in cell cultures. Evaluation of infected cultures can be facilitated by onducting enzyme-linked immunosorbent assays (ELISA) following a 1-2 day incubation, and nen scoring the extent of infection (and hence number of viable oocysts present in the original noculum) by spectrophotometry in an automatic plate reader. A detection sensitivity of pproximately 100 oocysts has been described (Upton *et al.*, 1994a; 1994b). Slifko *et al.* 1997 lescribe a semi-quantitative method which relies upon staining infected tissue cultures with luorescent antibody, and then tracking the numbers of infectious foci using epifluorescence and lifferential interference contrast microscopy. An adaptation of the tissue culture method using PCR (Rochelle *et al.*, 1997a) is described in Section VII-A-2 "Polymerase Chain Reaction" of this document.

3. Assessment of Laboratory Testing Capabilities

Detecting of *Cryptosporidium* and *Giardia* and distinguishing them from other organisms of comparable size and appearance is a major problem that presents most commercial, state, and local laboratories with a difficult challenge. However, it is important to establish whether these laboratories can follow a standard procedure and be successful in recovering and detecting these pathogenic protozoans in water samples.

Sixteen commercial laboratories were enlisted in a survey (Clancy *et al.*, 1994) to assess the ability of a laboratory to recover and detect *Giardia* and *Cryptosporidium* using the ASTM

method. Filters spiked with either *Giardia* (approximately 740) and *Cryptosporidium* (approximately 500) oocysts or with approximately 500 cells of *Oocystis minuta* (algal cells measuring 8-18 μm x 5-15 μm) were sent to the laboratories for analysis. Of the 12 laboratories that participated in the exercise, four reported *O. minuta* samples as positive for either *Giardia* or *Cryptosporidium*, while four others failed to recover *Giardia* from the cyst-spiked filter and six failed to recover *Cryptosporidium* from the oocyst-spiked filter. *Giardia* cyst recovery ranged from 0.8 to 22.3 % (with an average of 9.1%) while *Cryptosporidium* cyst recovery ranged from 1.3 to 5.5% (with an average of 2.8%). It was concluded that not all laboratories strictly followed the ASTM methods, and that the majority of laboratories need to improve in one or more areas: client response, quality of sampling equipment and directions for use, analytical methods, data accuracy, and reporting format. Expertise in microbiological identification also appeared to be lacking as indicated by the relatively high numbers of false positives. The EPA established an approval process for laboratories analyzing samples for the Information Collection Rule for *Cryptosporidium* and *Giardia*. The EPA required approved laboratories to have trained and experienced personnel performing *Cryptosporidium* and *Giardia* testing and the necessary processing equipment. In addition, initial and continuous passing of performance evaluation samples and passing of the on-site inspection were required.

B. Detection in Biological Samples

Diagnosis of *Cryptosporidium* infection is typically performed by examining the feces of an infected individual. Feces of patients with active cryptosporidiosis normally do not require concentration since oocysts are shed in great numbers; however, the number of oocysts can

fluctuate during the course of infection (Casemore and Roberts, 1993). This reiterates the importance of making diagnosis using multiple specimens. Concentration methods are employed and useful when trying to assess infection in immunocompromised patients with a history of unexplained diarrhea or in asymptomatic patients. Concentration methods including formalin-ether and formalin ethyl acetate sedimentation are commonly used in clinical laboratories. Sheather's sucrose floatation, zinc sulfate flotation, saturated sodium chloride floatation, discontinuous Percoll gradients, and cesium chloride gradient centrifugation are methods more common in research laboratories. Evaluations of various concentration techniques have been published and the results are summarized below.

Concentration methods

A study by Bukhari and Smith (1995), comparing water-ether, sucrose density gradient, and zinc sulfate concentration method, showed significantly higher numbers of oocysts were recovered from bovine feces using water-ether concentration. Rosales *et al.* (1994) used concentration by Sheather's solution to obtain greater numbers of oocysts than discontinuous Percoll gradients, and a commercially manufactured parasite concentrator device. Concentration of oocysts in cat feces (Mtambo, 1992) was best accomplished using formalin ether sedimentation which recovered 37% of the original oocysts compared to 11% and 33% for zinc sulfate and sucrose flotation, respectively. Clavel *et al.* (1996b) showed that simply increasing centrifugation times augmented oocyst recovery when using the standard formalin-ether acetate concentration method. Finn *et al.* (1996) demonstrated that straining the feces contributed to the loss of oocysts. This study showed a nearly four fold overall reduction in the number of oocysts detected

using a wash procedure.

Traditional staining methods

Traditional staining methods were described in the 1994 *Cryptosporidium* Criteria Document. Numerous new staining methods and variations of traditional methods have been employed. A review is included in Chapter 2 of the book, *Cryptosporidium* and Cryptosporidiosis (Fayer, 1997). Kang and Metham (1996) compared five staining methods for detection of *Cryptosporidium* oocysts. The safranin-methylene blue technique, a modified Ziehl-Neelsen method, was used as the "gold standard" and compared to two methods each using auramine and mepacrine stains with potassium permanganate and carbol fuschin as counterstains. The authors concluded that mepacrine and auramine staining procedures were easily performed and stained equally, however, they preferred mepacrine to auramine because it is less toxic and can be used with carbol fuschin without a decolorization step. Work by Ungureanu and Pontu (1992) supported these results. In the *Cryptosporidium* Screening Guidelines established by a joint working group, Casemore and Roberts (1993) recommended an auramine method to be used as a screening method with confirmation by a modified Ziehl-Neelsen. Acid fast staining methods do not stain all oocysts. Entrala *et al.* (1995) showed hydrogen peroxide treatment increased the percentage of oocysts displaying acid fast characteristics.

Immunofluorescence methods

The use of monoclonal antibody detection assays increases the sensitivity of *Cryptosporidium* detection. Garcia *et al.* (1987) tested 115 samples using a modified acid fast method and

monoclonal antibody staining. Sensitivity and specificity using the monoclonal antibody were 100%, while the acid fast method failed to detect 7 of 99 positive samples. Subsequent testing by Baron *et al.* (1989), Rusnak *et al.* (1989), Garcia *et al.* (1992), Tee *et al.* (1993), Grigoriew *et al.* (1994), Alles *et al.* (1995) and Roberts *et al.* (1996) confirmed these results. Studies using monoclonal antibodies for detection of *Cryptosporidium* in animals have also shown increased sensitivity (Wee *et al.*, 1995; Mtambo *et al.*, 1992; Xiao and Herd, 1993).

Enzyme Immunoassays

Commercial EIAs have been developed to replace time consuming microscopic methods. Most studies have shown EIA methods perform better than (Siddons *et al.*, 1991; Dagan *et al.*, 1995) or equal to (Chapman and Rush, 1990; Rosenblatt and Sloan, 1993; Parisi and Tierno 1994) conventional microscopic methods; however, work by Newman *et al.* (1993) indicated EIA methods were not sensitive enough to be used for patients without diarrhea. Enzyme immunoassays have been shown to be equal in sensitivity to the immunofluorescence assay (Siddons *et al.*, 1991; Rosenblatt and Sloan, 1993) and inferior using an experimental EIA (Anusz *et al.*, 1990) and a commercially available EIA (Ignatius *et al.*, 1997). Two commercial EIAs were compared to a direct immunofluorescence assay, the ProSpecT and the ColorVue (Aarnes *et al.*, 1994), and found to have differences in performance. The sensitivities were 96% and 72-76% respectively. Specificities were 97.6-99.5% using the ProSpecT and 100% using ColorVue.

Molecular methods: Polymerase Chain Reaction Assays

PCR-based assays are becoming more common and may provide a useful alternative for detecting and quantifying *Cryptosporidium* in water, stools, and tissue/organ samples. Awad-El-Kariem *et al.* (1994) described a PCR method that identifies *Cryptosporidium* at the species level and requires no DNA probes. The procedure is based on use of the published 18S rRNA genes of *C. parvum* and *C. muris*. One of the sequence 3 Mae I endonuclease restriction sites is present only on the *C. parvum* gene while others are specific for *C. muris* and *C. baileyi*, which allows screening for human, mouse, and avian species, respectively. The authors suggest the protocol they developed is adaptable to detection of small numbers of *C. parvum* oocysts in environmental samples. Morgan *et al.* (1996) noted the importance of PCR in processing clinical as well as environmental specimens suspected of being contaminated with *Cryptosporidium*. PCR primers specific for *Cryptosporidium* have been developed, and random amplified polymorphic DNA (RAPD) is a simpler approach for developing diagnostic primers since many of the products generated by RAPD-PCR are frequently species specific. Leng *et al.* (1996) developed an assay to identify *Cryptosporidium* DNA in bovine feces involving standardization of sample preparation and simplification of the DNA recovery process for PCR amplification and DNA-hybrid detection. The DNA-recovery/PCR detection procedure can recover DNA suitable for PCR amplification and can detect 10^3 to 10^4 times fewer oocysts in diluted solutions than the commercial enzyme-linked immunoassay, Color-Vue-*Cryptosporidium*, and 10^2 fewer from diarrhetic feces samples than the commercial Color-Vue kit.

Amplification methods have also been described which may assist in future efforts to define the role of livestock in waterborne outbreaks of cryptosporidiosis. Blassak *et al.* (1996) describe a rapid assay kit desgined to test fecal samples for live *Cryptosporidium* using a gene probe method. Oocyst DNA is released by cyclical freeze/thaw and is then amplified by isothermal strand displacement using biotinylated primers. The amplification product is detected colorimetrically in a microwell system in which a complementary capture probe binds to oocyst DNA and then reacts with a horseradish peroxidase-streptavidin conjugate. This complex emits a color that can be readily detected with qualitative or semi-quantitative results. The authors suggest that this method could be applied toward the analysis of genetic similarity among *Cryptosporidium* strains isolated from livestock and humans.

Balatbat *et al.* (1996) developed a nested PCR assay for detection of *C. parvum* directly from stool specimens. After extracting DNA from a formaldehyde-treated stool, a 400-bp fragment of DNA was amplified with two 26-mer primers. The amplicon from the first reaction was then subjected to a second round of amplification using a second set of primers. With these nested primers, a 194-bp fragment of DNA was amplified and confirmed as *C. parvum* DNA by internal probing with an enzyme-linked chemiluminescence system. The test can detect as few as 500 oocysts per gram of stool, and has the potential to detect asymptomatic infections, monitor response to therapy, or monitor environmental samples. The preliminary results indicate a significantly enhanced sensitivity compared with traditional assays.

Kelly *et al.* (1995) developed a sensitive and specific PCR test to confirm *Cryptosporidium*

infections. The test, which uses previously published primers to detect *Cryptosporidium* spp. in distal duodenal biopsies, was used to identify infections in HIV-positive patients in Zambia, and proved especially useful in identifying those infections that were limited to the distal small intestine.

Other methods

Serological methods have been used to monitor exposure to *Cryptosporidium*. There is limited information regarding the seroprevalence of *Cryptosporidium* infected individuals. The serologic response to *Cryptosporidium* is discussed in the section V-B and in Lengerich *et al.* (1993).

Flow cytometry methods have been described for detection of *Cryptosporidium* in mice (Arrowood *et al.*, 1995). More recent studies using seeded human feces showed a four-fold increase in detection over direct immunofluorescence methods (Valdez *et al.*, 1997). Chemiluminescence assays have been used by Clavel *et al.* (1996b) to detect cultured oocysts and other fecal parasites. You (1996) showed positive detection of *Cryptosporidium* in MDCK cell cultures and its potential for testing chemotherapeutic agents is promising. A reverse passive hemagglutination (RPH) assay (Farrington *et al.*, 1994) measuring agglutination of anti-oocyst antibody coated sheep erythrocytes with oocysts in diluted fecal suspensions was compared to auramine phenol staining for detection of *Cryptosporidium* and showed equal sensitivity.

C. Water Treatment Practices

Introduction

Iultiple barriers are used in most surface water treatment plants in an effort to prevent public xposure to waterborne pathogens like *Cryptosporidium*. These barriers include removal of athogens from water by processes like clarification and filtration, which are generally preceded y coagulation and flocculation processes. Another type of barrier is inactivation by isinfectants like ozone and chlorine. The purpose of this section is to summarize the removal nd/or inactivation of *Cryptosporidium* through multibarrier systems and through individual reatment processes. The reader is strongly encouraged to look at referenced studies to gain letailed information regarding site-specific raw water quality and treatment conditions.

n this section, log removal is given by:

$$\text{og removal} = -\log(N/N_0) \qquad \text{(Eqn. 2)}$$

where N is the concentration of *Cryptosporidium* oocysts remaining after treatment and N_0 is the concentration of *Cryptosporidium* oocysts prior to treatment. Log inactivation is given by a similar equation, but N and N_0 refer to the concentration of infectious *Cryptosporidium* oocysts in treated water and in untreated water, respectively.

2. Multi-Barrier Treatment

Several studies have evaluated the occurrence of *Cryptosporidium* in raw and finished waters for multibarrier treatment facilities (LeChevallier and Norton, 1995). In a survey of 72 North American drinking water plants, *Cryptosporidium* was present in 51.5 percent of raw water samples and in 13.4 percent of finished water samples. In an earlier survey of 66 drinking water plants, *Cryptosporidium* was observed in 87 percent and 27 percent of raw and finished waters,

respectively. The authors attributed the different occurrence levels between studies to normal variations in raw water quality and treatment performance. The authors claimed that microscopic analyses of *Cryptosporidium* oocysts in the finished waters suggested that most of the oocysts were nonviable. However, no attempts were made to specifically assess oocyst viability in this study. These occurrence results suggest that a significant percentage of *Cryptosporidium* oocysts are removed by drinking water treatment practices.

Other studies have reported removal of *Cryptosporidium* oocysts through conventional filter plants using chlorine as the primary disinfectant. The combination of coagulation, flocculation, and sedimentation achieved 3.8-log removal of oocysts in a treatment plant near Montreal, Canada (Payment and Franco, 1993). In the same treatment plant, 4.6-log removal of oocysts was observed through coagulation, flocculation, sedimentation, and granular media filtration (also known as conventional treatment). No attempts were made to assess oocyst viability in this study.

In a more comprehensive study of a conventional treatment plant near Pittsburgh, *Cryptosporidium* oocysts were detected in 63 percent of raw water samples, 29 percent of settled water samples, and 13 percent of filtered water samples (States, *et al.*, 1997). For those cases where oocysts were detected in both raw and settled waters, the treatment plant achieved 0.8 to 1.3-log removal of oocysts prior to filtration. For those cases where oocysts were detected in both raw and filtered waters, the treatment plant achieved 1.7 to 3.6-log removal of oocysts through filtration. Oocyst viability was not measured in this study, either.

3. Removal of *Cryptosporidium*

Introduction

As noted in the previous section, detectable *Cryptosporidium* concentrations occur infrequently in treated waters. Because of this, the effectiveness of treatment processes has been evaluated in challenge studies where oocysts are spiked into raw water at a concentration large enough to detect the oocysts in treated water. This section summarizes challenge study results for those processes that remove *Cryptosporidium* oocysts. Challenge study results for those processes that inactivate *Cryptosporidium* oocysts are summarized in the next section.

Coagulation, flocculation, and clarification

Several clarification methods are available for drinking water treatment and these are usually preceded by coagulant addition and flocculation. Currently, sedimentation is the most commonly practiced method of clarification in the United States. Other options include dissolved air flotation and sludge blanket clarification. One recent bench-scale study showed that dissolved air flotation was superior to sedimentation for removal of *Cryptosporidium* oocysts (Plummer, *et al.*, 1995). With proper coagulation and flocculation conditions, the combination of coagulation, flocculation, and dissolved air flotation could achieve 2.5 to 3.5-log removals of *Cryptosporidium*. Under similar conditions, the combination of coagulation, flocculation, and sedimentation could achieve no more than 1.0-log removal of oocysts. Similar bench-scale studies showed that 1.3 to 2.8-log removals of *Cryptosporidium* could be achieved by the combination of coagulation, flocculation, and dissolved air flotation (Hall, *et al.*, 1995).

Plummer, *et al.* (1995) observed relatively weak correlations between log removal of *Cryptosporidium* and percent removal of turbidity ($r^2 = 0.53$), UV absorbance ($r^2 = 0.52$), and dissolved organic carbon ($r^2 = 0.50$).

Coagulation, flocculation, sedimentation, and filtration (conventional treatment)

Pilot-scale treatment studies with two waters in the western United States showed that conventional treatment could obtain 3.0 to 6.2-log removal of *Cryptosporidium*, with a median of approximately 4.6-log removal (Patania, *et al.*, 1995). An average of 3.0-log removal was observed in another pilot-scale study in Utah, with a range of 1.9 to 4.0-log removal (Nieminski and Ongerth, 1995). Another Utah study was designed to evaluate *Cryptosporidium* removal in a full-scale treatment plant that was not delivering water to customers. In this case, removals ranged from 1.9 to 2.8-logs with an average of 2.3-logs (Nieminski and Ongerth, 1995). Actual performance depends on numerous factors including source water quality, chemical pretreatment conditions (e.g., coagulant dose and pH), filtration rate, bed depth, and filter media types. As noted earlier, the reader is advised to read referenced reports for more detailed information regarding the results summarized here.

The results to date suggest that the 2.0-log removal credit proposed for *Cryptosporidium* removal by conventional treatment is reasonable and, as shown by the above data, may be conservative. However, studies have only been published for waters from the western United States and need to be confirmed with results from other types of water supplies.

After combining data from the two Utah studies, Nieminski and Ongerth (1995) reported an r^2 of 0.79 for the relationship between log removal of *Cryptosporidium* and log removal of 4-7 μm sized particles. The correlation between log removal of *Cryptosporidium* and log removal of turbidity was weaker, with an r^2 of 0.55. Patania, *et al.* (1995) reported no significant correlations between log removal of *Cryptosporidium* and log removals of turbidity, 1-2 μm sized particles, 2-5 μm sized particles, and 5-15 μm sized particles. These latter results were obtained from a broader range of source waters and suggest that correlations between *Cryptosporidium* removal and removal of surrogate indicators may be site-specific. Further work is necessary before definitive conclusions can be reached. These two studies also based their conclusions on data obtained from different types of filtration practices (e.g., conventional treatment, direct filtration, in-line filtration). Further analysis is needed to determine whether their observations depend on the type of filtration practice.

Although no significant correlations were obtained between *Cryptosporidium* removal and turbidity removal, Patania, *et al.* (1995) observed that filter effluent turbidities less than or equal to 0.1 ntu were required to obtain 5-log *Cryptosporidium* removals on a reliable basis. Filter effluent turbidities of 0.2 ntu or less were needed to reliably maintain 4-log *Cryptosporidium* removals.

Coagulation, flocculation, flotation, and filtration

The effect of substituting sedimentation with dissolved air flotation on filtered water *Cryptosporidium* concentrations has not been directly compared in literature reports. However,

pilot-scale studies in the United Kingdom have shown that the combination of coagulation, flocculation, flotation, and filtration can achieve 2.9 to 4.[illegible]-log removal of oocysts (Hall, *et al.*, 1995). These results are consistent with those described above.

Coagulation, flocculation, and filtration (direct filtration)

The performance of direct filtration was assessed for the same Utah waters described in the conventional treatment section (Nieminski and Ongerth, 1995). For the pilot-scale system, *Cryptosporidium* removals ranged from 1.3 to 3.6-logs with an average of 3.0-logs. The full-scale system achieved an average oocyst removal of 2.8-logs with a range of 2.6 to 2.9-logs. Comparable removals were observed in pilot-scale studies with water from Seattle (Ongerth and Pecoraro, 1995). Results in this study showed 2.7 to 3.1-log removal of *Cryptosporidium*. Direct comparisons between direct and conventional filtration were only obtained with the pilot-scale system in Utah. Results indicate that there was no statistically significant difference between the two types of filtration. At this time, this conclusion can only be applied to this case and other studies will be necessary to determine those situations in which direct filtration achieves performance comparable to conventional treatment.

Coagulation and filtration (in-line filtration)

Using water from the Seattle water supply, pilot-scale treatment studies showed that in-line filtration could obtain 1.6 to 4.2-log removal of *Cryptosporidium*. The median value was approximately 2.8-log removal (Patania, *et al.*, 1995). Unfortunately, a direct comparison between in-line filtration and conventional treatment has not been made for the same water

supply.

Diatomaceous earth filtration

A recent study with water from Sydney, Australia concluded that diatomaceous earth filtration could perform significantly better than conventional treatment for removal of *Cryptosporidium* oocysts (Ongerth and Hutton, 1997). In this bench-scale study, removals ranged from 3.6 to 6.7 logs. Although these results appear to show superior performance of diatomaceous earth filtration, they were performed at filtration rates lower than those commonly used in conventional treatment facilities. Another study has shown comparable *Cryptosporidium* oocyst removals by pilot-scale diatomaceous earth filtration with a Pennsylvania water, where removals ranged from 4.6 to 5.9-logs (Schuler, *et al.*, 1991).

Slow sand filtration

Several studies have been performed to evaluate *Cryptosporidium* oocyst removal by slow sand filtration. Removals ranging from 3.9 to 7.1-logs were observed in pilot-scale studies with water from Pennsylvania (Schuler, *et al.*, 1991). Another study in the United Kingdom showed that oocyst removal exceeded 4.5-logs (Timms, *et al.*, 1995). Only 0.3-log oocyst removal was obtained in a slow sand filter located in British Columbia. However, the filter media in this filter did not meet standard specifications for slow sand filters (Fogel, *et al.*, 1993). Therefore, the results obtained to date suggest that slow sand filtration is an effective barrier to oocyst passage when the process is properly designed.

Membrane processes

In several microfiltration and ultrafiltration experiments, *Cryptosporidium* oocysts were not detected in treated waters when the membranes were intact (Jacangelo, *et al.*, 1995). This result was observed with feed oocyst concentrations as high as 9.1 x 10^4 oocysts/L, suggesting a removal capability of more than 7.1-logs. This result is expected because the pores within the membrane skin are smaller than *Cryptosporidium* oocysts. More recent studies have shown that *Cryptosporidium* oocysts could pass through a ceramic microfiltration membrane having a nominal porosity of 0.2 μm (Drozd and Schwartzbrod, 1997). In this case, oocyst removals ranged from 4.3 to 5.5-logs. However, no attempts were made to assess the integrity of the membrane and its associated equipment in this study.

4. Inactivation of *Cryptosporidium*

Introduction

The majority of studies performed to date have evaluated *Cryptosporidium* inactivation in buffered, demand-free waters and in small, batch reactors. Very little is known about the ability of alternative disinfectants to inactivate *Cryptosporidium* oocysts in natural waters or in flow-through treatment systems. Results depend on the method used to quantify inactivation and, if mouse infectivity is used to quantify inactivation, results may depend on the type of mouse and on the strain of *Cryptosporidium* used in the study. For these reasons, the reader is cautioned against the extrapolation of information presented in this section to inactivation of *Cryptosporidium* in natural waters and to infectivity in humans. The purpose of this section is to summarize the inactivation work performed to date for four disinfectant chemicals – ozone,

prechlorination (Finch *et al.*, 1997; Gyurek, *et al.*, 1997). Again, published reports are very preliminary and further studies will be needed to determine if this phenomenon is observed in natural waters and in other laboratories.

Mixed oxidants

A proprietary system that electrochemically produces a mixed oxidant solution was recently evaluated for its ability to inactivate *Cryptosporidium* oocysts (Venczel, *et al.*, 1997). This system was observed to achieve more than 3-log inactivation of *Cryptosporidium* oocysts with a 5 mg/L oxidant dose and a 4 hour contact time.

Ultraviolet radiation

Recent studies have demonstrated that some ultraviolet irradiation technologies may be promising for *Cryptosporidium* inactivation (Campbell, *et al.*, 1995; Arrowood, *et al.*, 1996). Because the technologies are not comparable, the results of these studies cannot be compared. In one case, ultraviolet irradiation produced 2 to 3-log inactivation of *Cryptosporidium* oocysts at ultraviolet doses and contact times achievable by commercial equipment (Campbell, *et al.*, 1995). In the other study, up to 6-logs of inactivation were observed with an alternative piece of commercial equipment (Arrowood, *et al.*, 1996).

D. Summary

Analysis of Water Samples

Filtration through wound yarn filters remains the most common collection method. Variations of

this collection methodology, including cellulose acetate membrane filters and capsule filters, could result in improved retention of oocysts, however, interlaboratory comparisons have not been published. Calcium carbonate flocculation methods, which can concentrate up to 10 liters of water, have also been shown superior to wound yarn filters but may interfere with viability determinations. Centrifugation-based concentration technologies such as vortex flow filtration, cross flow filtration, and continuous centrifugation could potentially recover greater numbers of oocysts than the currently used ASTM ICR methods, however the methods require interlaboratory validation. Immunomagnetic capture and flow cytometry also show considerable recovery increases using either seeded or environmental samples. However, performance will be influenced by water turbidity and composition. Laser scanning devices have also performed well in early studies, however more research is required. Several applications of the polymerase chain reaction for the detection of *Cryptosporidium* have been described in the literature, some of which may be able to distinguish viable from nonviable oocysts, however, enzymatic inhibition remains problematic. Since the determination of *Cryptosporidium* viability is critical in assessing the public health threat of cryptosporidiosis, a number of viability assays have been described and compared to animal infectivity models. In general, viability assays have produced conservative estimates of oocyst viability when compared to animal modeling data, however, limitations in viability assays have precluded their routine use in environmental samples. The USEPA has established an approval process for laboratories performing detection of *Cryptosporidium* and *Giardia* in water.

chlorine dioxide, chlorine, and monochloramine.

Ozone

Cryptosporidium inactivation by ozone has received a significant amount of attention. Ozone clearly achieves the most significant levels of *Cryptosporidium* inactivation when compared to the other disinfectants listed above (Korich, *et al.*, 1990; Finch *et al.*, 1997). In order to achieve 3-log inactivation of *Cryptosporidium* at pH 7, the product of contact time and ozone concentration (CT) needs to be in the range of 8 to 16 mg•min/L at 7°C and in the range of 3 to 15 mg•min/L at 22°C (Finch *et al.*, 1993). CT values for 2-log inactivation of *Cryptosporidium* by ozone at pH 7 are in the 5 to 10 mg•min/L range at 7°C and in the 2 to 8 mg•min/L range at 22°C. It is important to stress that these values are based on mean performance and do not account for the wide variability observed in test results. Results observed by others appear to be in general agreement when the variability in data is taken into account (Peeters, *et al.*, 1989; Korich, *et al.*, 1990; Parker, *et al.*, 1993; Owens, *et al.*, 1994a and b; Quinn, *et al.*, 1996; Finch *et al.*, 1997).

Chlorine Dioxide

Results obtained to date suggest that chlorine dioxide is the second most effective disinfectant on the above list (Peeters, *et al.*, 1989; Korich, *et al.*, 1990; Finch *et al.*, 1997, Liyanage, *et al.*, 1997a). CT values needed for *Cryptosporidium* inactivation by chlorine dioxide are considerably higher than those needed for ozone. To achieve a 1-log inactivation of *Cryptosporidium* at pH 7 and 25°C, CT values of approximately 60 mg•min/L would be necessary. At pH 8, the required

CT values appear to be greater than this. Recent results have shown that chlorine dioxide may be more effective if ozone is applied upstream of the chlorine dioxide addition point (Liyanage, *et al.*, 1997b). However, this practice is not likely to become common in the United States if currently proposed disinfection byproduct regulations are implemented.

Chlorine

At CT values commonly used in drinking water treatment, chlorine does not achieve more than a 1-log inactivation of *Cryptosporidium* oocysts (Korich, *et al.*, 1990; Fayer, 1995; Finch *et al.*, 1997; Gyurek, *et al.*, 1997; Venczel, *et al.*, 1997). This is true even in demand-free water at pH 6 and 22°C, conditions where chlorine can be expected to achieve the best level of performance. Recent screening studies have suggested that the ability of chlorine to inactivate *Cryptosporidium* may be enhanced by preozonation (Finch *et al.*, 1997). At this time, published reports are very preliminary and further studies will be needed to determine if this phenomenon is observed in natural waters and in other laboratories.

Monochloramine

Ever since the passage of the Surface Water Treatment Rule, monochloramine has not been frequently used as a primary disinfectant in drinking water treatment. Like chlorine, monochloramine is not capable of achieving more than a 1-log inactivation of *Cryptosporidium* at CT values commonly encountered in treatment practice (Korich, *et al.*, 1990; Finch *et al.*, 1997; Gyurek, *et al.*, 1997). Also, preliminary studies have shown that the ability of monochloramine to inactivate *Cryptosporidium* may be enhanced by preozonation or by

Analysis of Biological Samples

The 1994 *Cryptosporidium* Criteria Document describes the increased sensitivity of immunofluorescent antibody-based (IFA) procedures. Traditional staining methods such as the Ziehl-Neelsen stain, however, are still widely used. Enzyme immunoassay (EIA) methods are fast, inexpensive, easily performed, and show sensitivity approaching that of immunfluorescence methods. However, a lack of confirmatory analyses may preclude their routine use. Enzyme immunoassays may be useful for busy hospital laboratories or large scale screening surveys. Several PCR-based methods capable of distinguishing differences among specific strains have been described in the literature. As with testing the efficacy of different analysis of water methods, interlaboratory comparisons require strict adherence to oocyst quality and rigorous enumeration procedures. Recommendations by Klonicki *et al.* (1997) should be observed in future studies.

Summary of removal studies

Of the technologies available to the drinking water industry, membrane processes appear to provide the most significant levels of *Cryptosporidium* removal. Conventional treatment practices appear capable of meeting 2-log removals in most of the cases studied to date. Although direct filtration and in-line filtration may be expected to be less effective than conventional treatment, this has not yet been demonstrated in a conclusive manner. Alternative technologies like diatomaceous earth filtration and slow sand filtration appear capable of achieving comparable, if not better, levels of *Cryptosporidium* removal than conventional treatment.

Summary of inactivation studies

As noted above, ozone appears to be the best chemical disinfectant for *Cryptosporidium* inactivation at this time. The mixed oxidant and ultraviolet light systems appear to be promising, but have only been tested in minimal fashion when compared with ozone. Also holding some promise are the sequential disinfection systems of ozone followed by chlorine and ozone followed by monochloramine. Very few studies have evaluated *Cryptosporidium* inactivation in natural waters.

VIII. Research Requirements

A. Data Gaps

Many of the data gaps in our knowledge regarding *Cryptosporidium* previously identified in the 1994 *Cryptosporidium* Criteria Document have been filled, and an enormous amount of information has become available from research completed during the past 4 years. Many of the lessons learned from *Cryptosporidium* will be applicable to the next waterborne pathogen that emerges.

At a recent workshop on *Cryptosporidium* Issues related to drinking water, data gaps in four main areas of *Cryptosporidium* research, Source Water Occurrence, Analysis, Treatment, and Health Effects, were identified.

Source Water Occurrence: Although some data on the source and occurrence of *Cryptosporidium* in watersheds exist, more research is greatly needed. In particular, the fate and

transport of *Cryptosporidium* within watersheds is poorly understood. Improvements in monitoring methods and analytical techniques are greatly needed to increase our understanding of these issues.

Analysis: Research efforts for recovery of *Cryptosporidium* oocysts from water samples as well as from clinical samples have been improved and many of the steps in these processes that historically have been responsible for oocyst losses have been identified. Studies comparing methods have been done, and the advantages and disadvantages of various approaches elucidated. New detection methods are being developed, especially those using molecular biology approaches (e.g., PCR/gene probe procedures), laser-based technologies, and computer microscopy. Using these approaches, methods for determination of oocyst survivability in the environment and infectivity should improve significantly. Detection methods continue to be quite variable and the need for a standard method that is accurate, precise, quick and affordable still exists. Many of the newer technologies are yet unproven with real-world samples and validation testing must be completed. In addition, not enough is known about the basic cell biology of *Cryptosporidium*. Greater knowledge in this area will not only help in the development of an accurate detection method, but also advance viability, infectivity, and speciation assays for environmental *Cryptosporidium*.

Treatment: The need is great to develop, identify, and evaluate new methods for disinfection processes (e.g., ozonation, UV, improved filtration). In addition, compounds other than chlorine should be sought to serve as residual disinfectants in finished drinking water. Complete

evaluation of treatment for oocyst removal is dependent on better detection methods and more rigorous enumeration practices. Other gaps in the data regarding treatment of drinking water include: the usefulness and efficiency of surrogates to determine success of treatment, an understanding of the cellular biology and biochemistry involved in the treatment process, and guidelines or a decision matrix to assist in treatment selection.

Health Effects: Progress has been made in identifying compounds that can be used for human and animal therapy/treatment, although evaluation, validation, and clinical trials will be required. These drugs should be subjected to FDA approval after such research and clinical trials are completed. However, studies to develop new drugs should be continued. There has been very little progress in elucidating the pathogenic mechanisms involved in cryptosporidiosis, but U.S. EPA-sponsored human infectivity studies should provide some useful information. In addition to gaps in drug therapy, more information is needed to better identify and characterize outbreaks, to assess the risks to susceptible populations, and to identify the infectious dose and virulence across different populations. Finally, better diagnostic serological methods need to be developed and tested and more serology-based epidemiology studies need to be completed.

IX. References

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