Detection of Giardia lamblia and Cryptosporidium parvum in the eastern oyster (Crassostrea virginica) harvested from the Great Bay Estuary

Kim Marie Stowell
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Detection of Giardia lamblia and Cryptosporidium parvum in the eastern oyster (Crassostrea virginica) harvested from the Great Bay Estuary

Abstract
The Great Bay Estuarine system is being impacted by point source and nonpoint source pollution that affect shellfish quality. Many outbreaks of human gastroenteritis are potentially caused by the consumption of raw or undercooked oysters in which the protozoan pathogens, Giardia lamblia and Cryptosporidium parvum are present. The development of a rapid and sensitive technique to detect infectious protozoa in oysters is a necessity.

Immunofluorescence has been employed as a technique for the detection of these organisms in drinking water. Cross reactivity with algae and the production of false positive results limits the usefulness of this technique in environmental studies. Molecular approaches, such as the polymerase chain reaction, are often overlooked in environmental studies. This is due to obstacles such as enzymatic inhibitors that are intrinsic in the environment as well as the small sample size that can be analyzed.

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To determine the applicability of this method to field studies, three sites were sampled in the Great Bay Estuarine system using a sample size of 25 oysters. Two of the selected field sampling sites were classified as approved for shellfish harvesting based on most-probable-number water analysis and one site was closed to shellfish harvesting, due to high fecal coliform counts (>70 fecal coliforms/100 ml water). Each site sample was processed and analyzed using the multiplex PCR and nested PCR procedure. The presence of Cryptosporidium parvum and/or Giardia lamblia was detected at each field sampling site.

Keywords
Biology, Microbiology, Biology, Ecology, Agriculture, Fisheries and Aquaculture

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DETECTION OF *GIARDIA LAMBLIA* AND *CRYPTOSPORIDIUM PARVUM* IN THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) HARVESTED FROM THE GREAT BAY ESTUARY

BY

Kim M. Stowell
B.S., University of New Hampshire, 1995

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Microbiology

September, 2001
This dissertation has been examined and approved.

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5/3/01
Date

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DEDICATION

This is dedicated to my husband and to my parents, for all their love and support. What a long strange trip it's been.
ACKNOWLEDGEMENTS

I would like to thank my committee members Dr. Stephen Torosian, Dr. Aaron Margolin, Dr. Thomas Pistole, Dr. Robert ZsigRAY and Dr. Stephen Jones for their help throughout my years at UNH.
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ABSTRACT

DETECTION OF GIARDIA LAMBLIA AND CRYPTOSPORIDIUM PARVUM IN THE EASTERN OYSTER (CRASSOSTREA VIRGINICA) HARVESTED IN THE GREAT BAY ESTUARY

by

Kim M. Stowell
University of New Hampshire, September, 2001

The Great Bay Estuarine system is being impacted by point source and nonpoint source pollution that affect shellfish quality. Many outbreaks of human gastroenteritis are potentially caused by the consumption of raw or undercooked oysters in which the protozoan pathogens, Giardia lamblia and Cryptosporidium parvum are present. The development of a rapid and sensitive technique to detect infectious protozoa in oysters is a necessity.

Immunofluorescence has been employed as a technique for the detection of these organisms in drinking water. Cross reactivity with algae and the production of false positive results limits the usefulness of this technique in environmental studies. Molecular approaches, such as the polymerase chain reaction, are often overlooked in environmental studies. This is due to obstacles such as enzymatic inhibitors that are intrinsic in the environment as well as the small sample size that can be analyzed.

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INTRODUCTION

Waterborne Disease

Waterborne illnesses caused by enteric pathogens are problematic in the United States. Enteric organisms are those pathogens that replicate in the intestinal tract of warm-blooded animals; they are transmitted throughout the environment via the fecal-oral route (Hortsmann, 1967). Waterborne pathogens cause gastroenteritis in the human population via ingestion of contaminated drinking water, water products, or shellfish. In addition, epidemiological data on waterborne disease surveillance is often limited and underestimates the burden of disease, making risk assessment difficult to determine (Craun et al., 1996; Frost et al., 1996).

Typically waterborne illness has been attributed to viral and bacterial pathogens that have contaminated water and food (Clayman, 1989). However, there are several outbreaks with unknown etiologies, showing that the cause of the illness was unidentified possibly due to the lack of appropriate assays and testing techniques (CDC, 1990). Some of these previously unrecognized infectious agents became identified when protozoans were recognized as being capable of being transmitted by the waterborne route. The number of waterborne illnesses with unknown etiologies declined with an increase in this recognition (CDC, 1990). The Department of Health and Human Services has identified two significant protozoan pathogens, *Giardia lamblia* (*G. lamblia*) and *Cryptosporidium parvum* (*C. parvum*). National reporting of *G. lamblia* as a human pathogen began in 1986 and *C. parvum* began in 1995. Since then there have been documented outbreaks of each, both waterborne and foodborne.
Giardia *lambila*

*G. lambila* is a protozoan pathogen of human concern. It is approximately 8-12 μm in size and is found as an inactive, quadrinucleated, ovoid cyst in the environment (Ongerth, 1987). *Giardia* is a primitive eukaryote, unicellular and lacking mitochondria and peroxisomes (Soltys, 1999). Until recently it was thought that *Giardia* also lacked an endoplasmic reticulum and Golgi apparatus (Soltys, 1999).

The life cycle of *Giardia* alternates between a trophozoite and a cyst. Once ingested by a host, the cyst excysts upon exposure to the gastric juices of the stomach (Gillin et al., 1996). The emerging trophozoite must not completely emerge until the cyst passes into the small intestine or it will be killed by the low pH of the stomach (Soltys, 1999). The parasite begins asexual reproduction by rapidly dividing into two binucleate trophozoites (Soltys, 1999; Gillin et al., 1996). The trophozoite is motile via four pairs of flagella, allowing the organism to infiltrate the mucosa and attach to the intestinal epithelia using their ventral adhesive disc (Soltys, 1999; Adam, 1991; Friend, 1966; Nemanic et al., 1979). The trophozoite is the life stage of *Giardia* that produces the symptoms of infection. The environment beyond the small intestine is inhospitable to the trophozoite and therefore it must encyst to survive (Gillen et al., 1996). The most visible change in the cell during encystation is the detachment and rounding of the trophozoite (Gillen et al., 1996). This pre-encysted organism loses motility and becomes refractile (Gillen et al., 1996). The cyst wall is then completed and the organism is released back into the environment through shedding in the host feces.

Humans are capable of harboring a wide diversity of *Giardia* isolates, many of which are morphologically identical to those found in other mammalian species (Nash,
1995). This led to the speculation that many different animals can serve as reservoirs for human infection (Nash, 1995).

*Giardia* species are able to undergo surface antigenic variation *in vitro* (Adam, 1991; Bruderer et al., 1993). This variability could directly affect the host-parasite relationship, since *Giardia* surface proteins are thought to be targets for the host immune response (Nash, 1995). By expressing new epitopes over time, the duration of infection may be lengthened (Nash, 1995). It has been shown that antigenic variation does occur *in vivo* and that the initial surface protein epitope remains unchanged until day 14 of the infection (Nash, 1995). By day 22, the original epitope has been completely replaced by a mixture of other surface proteins (Nash, 1995). Antigenic variation in *Giardia* occurs in neonatal nude mice, which lack a cellular immunity (Gottstein et al., 1991). This has led to the hypothesis that the humoral immune system influences the variation in surface proteins (Gottstein et al., 1991).

The *Giardia* cyst is able to resist many natural environmental stresses, including desiccation, ultraviolet light, fluctuations in pH, and enzymatic degradation as well as disinfection processes such as chlorination (Fayer et al, 1997). *G. lamblia* is able to bring about gastrointestinal illness, giardiasis, characterized by severe attacks of diarrhea (Clayman, 1989). Symptoms can range from asymptomatic to severe, with the most severe infections potentially resulting in intestinal blockage. Symptoms typically last for 1-2 weeks, although cases of chronic giardiasis have been noted (Ash et al., 1997). Giardiasis can result following the ingestion of as few as one to 10 cysts (Ongerth, 1987). This infectious dose is extremely low, implying enhanced virulence.
*G. lamblia* is the most common cause of nonbacterial diarrhea in North America (Ash et al., 1997). Studies on sampled water sites in the United States have found *Giardia* cysts in 45% of surface water and 6% of groundwater (Sykora et al., 1988; LeChevallier et al., 1995; Rose et al., 1996; Hancock et al., 1998; Gibson et al., 1998). Outbreaks of foodborne giardiasis include 48 documented cases occurring in the United States in 1989-1990 due to contaminated vegetables. Multiple outbreaks of waterborne giardiasis occurred in the United States between 1991-1994. These outbreaks were the result of drinking water (508 cases) and recreational water (175 cases) (Osterhom et al., 1981; Peterson et al., 1988; White et al., 1989; Porter et al., 1990; Quick et al., 1992; Mintz et al., 1993; Smith, 1993; CDC, 1993, 1996, 1997).

Identifications of infection caused by *G. lamblia* have increased from the 4.0% average found in 1979 (CDC, 1992). Forty states have reported increases in *G. lamblia* infections and seven reported decreases (CDC, 1992). *G. lamblia* infections increase in the summer and fall, especially in northern states generating a seasonal outbreak pattern (CDC, 1992). Sources of infection include warm-blooded animals, in particular beavers. The most common way to acquire giardiasis is by drinking contaminated water or by eating contaminated food products.

*Cryptosporidium parvum*

Another protozoan associated with disease in humans is *C. parvum*. This organism was classified as a nationally notifiable disease in 1995 and is considered to be an emerging pathogen (CDC, 1998).
*C. parvum* is found in environmental waters as a small, 3-7 μm spherical double membrane oocyst (Holley, 1986). Each oocyst contains four motile sporozoites which are 5 by 1 μm in size (Clark, 1999). Once the oocyst is ingested, the sporozoites are released and the infection process begins. Sporozoites attach to and invade the intestinal epithelial cells of the host (Clark, 1999). It is thought that this invasion is the result of a discharge of molecules from the parasite organelles (rhoptries, micronemes and dense granules), which are located at the apical end of the sporozoite (Clark, 1999). The release of the molecules disrupts the microvilli of the epithelial cells and allows the parasite to enter, enveloping itself in the host membrane during the process (Clark, 1999).

*Cryptosporidium* then completes its asexual replication by replicating into eight merozoites (Clark, 1999). The merozoites are then released from the cell and are able to infect neighboring epithelial cells (Clark, 1999). The merozoites then differentiate into gamonts, which undergo sexual reproduction to regenerate oocysts (Clark, 1999). These oocysts are then released back into the environment in the feces of the host (Clark, 1999; Current, 1985).

Gastrointestinal disease, cryptosporidiosis, is acquired upon ingestion of approximately 15 oocysts. Symptoms, including profuse diarrhea, typically occur for five to 11 days (Holley, 1986). The infection is usually self-limiting in healthy individuals. In immunocompromised individuals, this illness can be life-threatening, leading to the loss of up to 17 liters of fluid per day (Leng, 1996). Although the intestinal tract is the primary site of cryptosporidiosis, other organs have been implicated in AIDS-associated *Cryptosporidium* infections, including the lungs, middle ear, biliary tract, pancreas and stomach (Dunand et. al., 1997; French et. al., 1995; Rossi et al., 1998).
developing countries, children are most susceptible to cryptosporidiosis. It has been noted that children under the age of one year are most susceptible and that this disease is associated with malnutrition (Lima et al., 1992, Macfarlane et al., 1987, Molbak et al., 1997). Children diagnosed with symptomatic cryptosporidiosis grew less in the first month of infection as compared with those children who were not infected (Clark, 1999).

There have been many outbreaks in the United States attributed to C. parvum. One such outbreak occurred in Milwaukee, Wisconsin where over 400,000 people became ill from drinking water from a contaminated public drinking water supply (MacKenzie et al., 1995). Contaminated recreational water was responsible for outbreaks involving 1218 cases in the United States (1991-1994). Foodborne infections include 185 cases in 1993 and 1996 due to contaminated apple cider and 15 cases in 1995 due to contaminated chicken salad (Osterhom et al., 1981; Peterson et al., 1988; White et al., 1989; Porter et al., 1990; Quick et al., 1992; Mintz et al., 1993; Smith, 1993; CDC, 1993, 1996, 1997). Studies have shown that 51.5% of sampled source waters and 11% of ground water in the United States contained Cryptosporidium oocysts (Sykora et al., 1988; LeChevallier et al., 1995; Rose et al., 1996; Hancock et al., 1998; Gibson et al., 1998).

Reservoirs of infection of Cryptosporidium species include ruminants (Fayer et al., 1998), Canadian geese (Fayer et al., 1998), domestic dogs (Morgan et al., 2000), feral pigs (Atwill et al., 1997) and house flies (Graczyk et al., 1999). This organism has been of particular concern to the drinking water industry due to its ability to resist typical disinfection methods such as chlorination (Robertson et al., 1992). Many environmental stresses, including freezing at -20°C, do not render this organism noninfectious (Fayer et
In 1998, the total number of reported cases for cryptosporidiosis in the United States was 3,793, with the largest number of cases seen in the month of August (CDC, 1998). National reporting for cryptosporidiosis began in 1995 with 2,972 cases reported from 27 states. During 1996, as cryptosporidiosis became a reportable disease in an increased number of states, 2,426 cases were reported from 42 states. In 1997, a total of 2,566 cases were reported from 45 states. Since the diagnosis of cryptosporidiosis is often not considered, and laboratories do not routinely test for Cryptosporidium infection, cryptosporidiosis continues to be underdiagnosed and underreported (CDC, 1997).

Protozoans in the Environment

C. parvum and G. lamblia are widespread in the environment, with one or both of these protozoans found in 97% of raw waters tested (LeChevailler, 1991). These organisms were primarily considered to be freshwater pathogens. However, it has been noted in recent years that these organisms are able to withstand saline environments. One study showed that Cryptosporidium oocysts are able to survive and remain infectious when incubated in seawater for 12 weeks (Fayer et al., 1998). A subsequent study demonstrated viable and infectious oocysts for up to one year in similar conditions (Tamburrini et al., 1999). For this reason there is a need for an assay to determine the presence of Giardia and Cryptosporidium in shellfish.

These organisms are transmitted via the fecal-oral route and are introduced into waterways primarily from three different sources; direct human fecal contamination, animal reservoirs, and farm runoff (Figure 1) (CDC, 2000; Milne, 1989). The numerous sources and reservoirs of infection for these pathogens impact a large portion of the environment. The impact is cyclic, particularly with human contamination (Figure 1).
FIGURE 1. Sources and Reservoirs of Protozoan Infection in the Environment

(adapted from Milne, 1989)
Regular monitoring of water can be used to detect contamination from water treatment systems, recreational water and effluents discharged both in water and on land. Major epidemics are often the result of infected livestock, wildlife and humans, which amplify the pathogens and release them in larger numbers back into the environment. Localized outbreaks of Giardia and Cryptosporidium are seen with humans, particularly in children in day care facilities (Milne, 1989).

While outbreaks of G. lamblia and C. parvum are continually documented, exposure to low concentrations of cysts and oocysts may be associated with endemic levels of disease (Gibson et al., 1998). Techniques used in epidemiology may not be sensitive enough to detect these low concentrations. This may result in the inability to thoroughly evaluate the risks associated with these organisms (Gibson et al., 1998). Risks may not be fully defined since the organisms are able to cause varying degrees of stress to the host, ranging from asymptomatic to severe (Gibson et al., 1998).

The Great Bay Estuarine System

The Great Bay Estuarine system consists of 5,280 acres, including 4,471 acres of tidal waters and mudflats and approximately 48 miles of shoreline (Short et al., 1992). The system is made up of the Piscataqua River, Little Bay and Great Bay (Figure 2) and is known as one of the most recessed estuaries in the nation, since the ocean and river waters mix 15 miles inland from the Atlantic Ocean (Short et al., 1992).
FIGURE 2. The Great Bay Estuarine System, New Hampshire

(Adapted from Nelson, 1982)
An estuary allows for the diversification of species due to the salinity and temperature differences between the saline ocean and incoming freshwater from rivers. This mixing produces brackish water with moderate salinity levels. The relatively shallow water also allows for increased temperatures in the Great Bay as compared with the ocean. These factors allow a variety of creatures to flourish and provides protection for spawning marine organisms.

The Great Bay Estuarine System provides a haven for over 281 species of birds, including wintering waterfowl (Short et al., 1992). The diverse landscape allows for easy migration of many types of animals, allowing populations to flourish.

It is also a haven for fisherman and shellfish harvesters. With the closure of other shellfish beds due to pollution, Great Bay remains the area most heavily harvested for oysters (Short et al., 1992). Oyster harvesting is a popular recreation for many New Hampshire residents. New Hampshire allows for recreational harvesting and residents are able to harvest oysters year round, with the exception of July and August, and may harvest up to one bushel per day (NH Fish and Game, 2000). The recreational harvesting of oysters continues to grow in popularity, thus making the problem of contamination a priority.

**Points of Pollution**

There are several factors that contribute to pollution into the Great Bay Estuarine System. Point source pollution in the Estuary includes sewage treatment plants, combined sewers, direct discharge, and industry (Table 1) (Short et al., 1992; NHEP, 2000).
TABLE 1. Rivers Affected by Treatment Plants that Flow into the
Great Bay Estuary

<table>
<thead>
<tr>
<th>RIVER NAME</th>
<th>TREATMENT PLANT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamprey River</td>
<td>Newmarket</td>
</tr>
<tr>
<td>Squamscott River</td>
<td>Newfields, Exeter</td>
</tr>
<tr>
<td>Oyster River</td>
<td>Durham</td>
</tr>
<tr>
<td>Piscataqua River</td>
<td>Dover, Newington, Kittery, Portsmouth</td>
</tr>
<tr>
<td>Bellamy River</td>
<td>NA</td>
</tr>
<tr>
<td>Salmon Falls River</td>
<td>Somersworth, Berwick</td>
</tr>
<tr>
<td>Cochecho River</td>
<td>Rochester</td>
</tr>
</tbody>
</table>

NA: not applicable
Other contributors of pollution into the Estuary include urban runoff, agricultural runoff, and wildlife (Short et al., 1992; NHEP, 2000). Beavers, known as a major contributor of *G. lamblia* to the environment, are residents of the Great Bay Estuary along with a variety of seabirds and waterfowl.

Waterfowl and seabirds, including great blue herons and gulls (Fayer et al., 1996) have been implicated in spreading both *G. lamblia* and *C. parvum* into the environment (Graczyk et al., 1998). Populations of wintering Canadian geese in the Estuary have been estimated to be up to 900 geese during the winter months (Short et al., 1992). Other seabirds and waterfowl such as black ducks are abundant in the Bay and may be a contributing factor to the spread of *Giardia* and *Cryptosporidium*.

There have been numerous studies done on the presence of viral and bacterial contaminants in water (Short et al., 1992; Burkhardt et al., 2000; Cabelli et al., 1970; NHDES, 2000). However, relatively little is known about *G. lamblia* and *C. parvum* in estuarine environments and their uptake by oysters.

**Oyster Safety**

Oysters are in the class Pelecypoda (Lamellibranchia) and order Filibranchia. They possess attenuated, reflexed gill filaments. The filaments are incompletely fused; with interlamellar junctions present but adjacent filaments joined only by ciliary tufts (Galstoff, 1964). Oysters are filter feeders and are able to concentrate fecal contaminants along with food particles (Galstoff, 1964; NOAA, 1991). If pathogenic organisms are present in the water column, they may accumulate in the shellfish at levels considerably higher than in the overlying water (Metcalf et al., 1979; Rippey, 1994). The rates and
kinetics of bioaccumulation and elimination of enteric viruses and bacteria depend on a variety of conditions; the shellfish species (Cabelli et al., 1970), the type of microorganism (Burkhardt et al., 1992) as well as the environmental conditions and season (Burkhardt et al., 1992; Cabelli et al., 1970).

Typically, molluscan shellfish are studied based on bacterial and viral contamination, including microorganisms such as *Salmonella typhi*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, hepatitis A virus and Norwalk-like virus (Burkhardt et al., 2000). It has been well documented that illness from ingestion of undercooked shellfish resulted in more than 2,100 cases of intestinal disease in the United States between the years of 1991 to 1998 (Burkhardt et al., 2000).

In 1925, the National Shellfish Sanitation Program (NSSP) was developed following a widespread outbreak of typhoid fever in which 1,500 cases and 150 deaths were reported (Rippey, 1994). The outbreak was traced to sewage-polluted oysters (Rippey, 1994). The role of the National Shellfish Sanitation Program is to prevent shellfish-associated illnesses, specifically caused by enteric bacteria (Frost, 1925). Outbreaks of shellfish-borne gastroenteritis are still seen despite the National Shellfish Sanitation Program’s regulations.

In the following statement of the purpose of the National Shellfish Sanitation Program, one can see there is no mention of protozoa.

The National Shellfish Sanitation Program assumes that a relationship exists between pollution from human activities, shellfish growing waters and human disease. Pathogens (disease-causing bacteria or viruses) may enter waters through direct discharges of untreated or poorly treated human wastes or through nonpoint runoff from streets, farms or construction sites. Bivalve molluscs, such as oysters, filter large volumes of water and concentrate pollutants and pathogens (The 1990 National Shellfish Register of Classified Estuarine Waters).
Shellfish harvesting sites are classified as approved, conditionally approved, conditionally restricted, or prohibited based on fecal coliform counts (NOAA, 1991). In order for waters to be approved for recreational shellfish harvesting, water samples collected at low tide must achieve a most probable number (MPN) of fecal coliform bacteria lower than 14/100 ml (NHEP, 2000). It has been noted, however, that fecal coliforms may not be adequate indicator organisms as they are rendered noninfectious and/or nonviable easier than other enteric pathogens (Grimes, 1987).

Some scientists and fishermen also question the use of fecal coliform concentrations for classifying shellfish growing areas, particularly in waters that receive chlorine-disinfected effluents from wastewater treatment plants (Rippey, 1994). Although chlorine is generally effective in inactivating most bacterial species, including bacterial pathogens, it is less effective against enteric viral pathogens found in sewage, such as the common Norwalk-type virus associated with minor intestinal distress (Rippey, 1994). *Giardia* cysts and *Cryptosporidium* oocysts are also unresponsive to treatment with low levels of chlorine, allowing for accumulation in oysters. The use of alternative indicators of fecal pollution is currently under investigation by the Food and Drug Administration.

Current diagnostic procedures used in clinical laboratories may be inadequate for the detection of *Giardia* and *Cryptosporidium* in oysters. False positive results have been reported using *Cryptosporidium* antibodies in an enzyme-linked immunosorbent assay (CDC, 1999). From November 1997 to March 1998, five states reported problems with the assay, resulting in approximately 225 samples that produced either false positive or indeterminate results (CDC, 1999).
A method currently used for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water and shellfish is the immunofluorescence assay. This method utilizes antibodies specific for *Giardia* species and *Cryptosporidium* species and requires microscopy to distinguish between the two organisms based on morphological characteristics. The concern with using this technique when analyzing environmental samples is that the antibodies may antigenically cross-react with various species of algae (Rodgers et al., 1995). This may lead to a potential public health hazard.

Infectious *C. parvum* has been found in oysters (Graczyk et al., 1998), cockles (Gomez-Bautista et al., 2000), and mussels (Gomez-Bautista et al., 2000). *G. lamblia* and *C. parvum* have been detected in freshwater clams (Graczyk et al., 1997) and estuarine clams (Graczyk et al., 1999). Studies on *C. parvum* have shown that oocysts are present in the hemocytes, on the gills and in the intestinal track of oysters (Graczyk et al., 1997). With optimum feeding conditions, a mid-size clam has been able to retain, by hemocyte internalization alone, an average of $3.68 \times 10^6$ oocysts (Graczyk et al., 1997). Studies have proven that one week after ingestion, oocysts retain their infectivity in mice (Fayer et al., 1998).

Filtration rates for adult oysters range from approximately 3 liters per hour to as high as 34 liters/hour (Galstoff, 1964). The filtration rate depends on environmental conditions such as water movement, salinity and temperature (Galstoff, 1964). Oysters are euryhaline organisms and are able to live in two general saline zones, polyhaline (30-18 ppt) and mesohaline (18-5 ppt) (Galstoff, 1964). The temperature at which the oyster ceases feeding is 6-7° C while maximum ciliary rate is seen at temperatures of 25-26° C (Galstoff, 1964). Temperatures above 32° C result in rapidly declining ciliary
movement (Galstoff, 1964). By observing seasonal fluctuations in temperature and salinity, it’s possible to monitor times of reproduction and feeding (Galstoff, 1964). These environmental conditions are representative of seasonal conditions observed in the Great Bay Estuarine System.

New Hampshire Oysters

In New Hampshire, all harvest-limited waters are adversely affected by sewage treatment plants (Table 1) (Short et al., 1992, NHDES, 2000; NHEP, 2000).

Harvest-limited waters are also significantly affected by industry and agricultural runoff. The effects of these sources have required the State of New Hampshire to close or restrict 64% of its classified shellfish growing waters due to the presence of enteric pathogens, defined as human intestinal bacteria or viruses that cause gastroenteritis or hepatitis (NOAA, 1991).

There has been no commercial harvesting of shellfish in New Hampshire since 1986 (Short et al., 1992). Only recreational harvesting is allowed in approved shellfish-growing waters. The State of New Hampshire estimates that downgrades of shellfish-growing waters and harvest restrictions over the last 20 years have resulted in an 85% loss in harvestable softshell clams and a 67% loss in harvestable oysters (NOAA, 1991).

Thus rapid and reliable detection of human pathogens in oysters is necessary. A detection system can be utilized with techniques that directly detect the pathogens rather than complete reliance on indicator organisms to predict the presence or absence of other enteric pathogens. Obstacles to molecular assays used in environmental studies typically include a limited sample size for analysis as well as enzymatic inhibitors that are inherent
to the environment. These obstacles are surmounted using a technique such as immunomagnetic separation coupled with multiplex PCR.

**Immunomagnetic Separation**

A novel approach to recovering *Giardia* cysts and *Cryptosporidium* oocysts from sample concentrates is through the use of immunomagnetic bead technology. Immunomagnetic separation (IMS) has been used widely in potable water samples with recovery efficiencies up to 95% (Dynal, 1997). Recovery rates of cysts and oocysts from environmental water samples vary depending on turbidity (Deng et al., 1997). In addition to *Giardia* and *Cryptosporidium* other organisms that have been recovered using IMS technology include enteric viruses and *Helicobacter pylori* from fresh water (Bifulco et al., 1993; Enroth et al., 1995); *Pseudomonas stutzeri* from seawater (Bard et al., 1997); and *Salmonella* species, *Mycobacterium avium*, and enterohemorrhagic *Escherichia coli* from fecal samples (Chapman et al., 1997; Kongmuang et al., 1994).

Although IMS use is expanding in environmental work, its effectiveness is often limited in oysters due to the dense tissue mass that prevents optimum binding of the beads to their target. As a result, detection of pathogens in shellfish using an antibody-coated bead technique requires additional manipulation of the sample by removing specific portions of the oyster for analysis (Graczyk et al., 1998, Gomez-Bautista et al., 2000). This approach, while reducing the amount of tissue that would inhibit binding of the beads, also limits the amount of oyster to be analyzed. This research utilized an ethyl acetate sedimentation step prior to IMS to debulk the sample, allowing for the entire oyster to be analyzed.
The IMS method provides a rapid and selective means to remove Giardia species and Cryptosporidium species by utilizing paramagnetic beads linked to antibodies specific for surface antigens found on the Giardia species cyst or Cryptosporidium species oocyst. The captured cysts/oocysts are held stationary while repeated washing steps remove the surrounding matrix. Therefore this technique greatly reduces the concentration of substances that may be inhibitory to subsequent molecular manipulations (Dynal, 1997). The processed sample is also concentrated allowing for a greater portion of the original sample to be included per polymerase chain reaction (Gomez-Bautista, 2000).

**Multiplex Polymerase Chain Reaction**

The polymerase chain reaction (PCR) is a nucleic acid amplification procedure in which DNA sequences are exponentially amplified. PCR utilizes oligonucleotide primers that are complementary to specific regions of the DNA sequence, allowing researchers to amplify genes of interest. The PCR process involves temperature cycling to denature the DNA, to anneal the primers and to form DNA via extension of the primers, catalyzed by a thermostable DNA polymerase.

Multiplex PCR has the advantage of simultaneously amplifying multiple loci (Morgan et al., 1998). An additional benefit to using multiplex PCR is the addition of a universal primer sequence (UPS) to the 5’end of the oligonucleotide primers, making them chimeric (Shuber, et al., 1995). A UPS that is used with consistent results is a 20-mer sequence taken from the bacteriophage M13mp18 (Shuber et al., 1995). This sequence functions to stabilize the kinetics of the process, allowing for fewer
optimization steps that are normally indicative to the development of a multiplex procedure (Shuber et al., 1995). This reduces the overall cost and effort involved in the detection process.

PCR has been used to detect viruses in shellfish (Atmar et al., 1993; Lees et al., 1994); however, approach to detect *G. lamblia* and *C. parvum* in shellfish currently utilizes the immunofluorescence assay (IFA). There are two main concerns when applying the IFA method to environmental samples. It has been reported that the IFA produces false positive results with algae (Rodgers et al., 1995). There is also a concern with using this technique since there is antigenic variability observed with *G. lamblia* (Nash, 1995) and *C. parvum* (Griffin, 1992). Comparative trials have shown that PCR has been determined to be more sensitive, specific and accurate than immunoassays for the detection of protozoan pathogens in both fecal samples and water samples (Morgan et al., 1998). In addition, by targeting specific genes, multiplex PCR allows for the simultaneous detection of *G. lamblia* and *C. parvum*, the human pathogens. Utilization of specific methods such as multiplex PCR would allow for further information to be gathered to help develop risk assessment models for *G. lamblia* and *C. parvum* in oysters.

**Risk Assessment of Giardia lamblia and Cryptosporidium parvum in Oysters**

Risk assessment is defined as a systematic process for qualitative or quantitative characterization of adverse effects associated with hazardous substances, processes, actions and/or events (NRC, 1983; Covello et al., 1993). The approach to risk assessment is made up of four components; hazard identification, dose response, exposure assessment and risk characterization.

20
The goal of hazard identification is to identify the pathogen and to determine the risk of ingesting that pathogen (DiNovi, 2000; Gibson, 1998). This risk is determined using data from previous outbreaks and/or animal models to study the severity and duration of the illness (DiNovi, 2000).

The dose response model looks at the interactions of the pathogen with the host and its environment (Burr, 2000). Several uncertainties develop when devising a model for *G. lamblia* and *C. parvum* in oysters. Some of the uncertainties for the host include predisposing factors, such as immunocompetency, making the host more or less susceptible to the pathogen. The population most susceptible to enteric microorganisms includes the young, malnourished, elderly, disease-impaired and a broad category of immunocompromised individuals (AIDS patients, transplant recipients and those on chemotherapy) (Gerba et al., 1996). In 1997, it was estimated that the immunocompromised population represented 20% of the total population in the United States and this population is predicted to increase in the years to come (Rose, 1997).

Varying degrees of disease severity complicate risk analysis. Uncertainties associated with the pathogen include the severity of illness and the consistency of introduction of the pathogen into the environment. A major uncertainty in the distribution of these pathogens in the environment is that depuration rates are unknown at this time for *G. lamblia* and *C. parvum* in oysters. The environment is also a significant component to the oysters' depuration ability, with variable factors such as temperature and salinity affecting the uptake and release of these organisms by oysters (Galstoff, 1964). Some data are available using human subjects to determine factors when developing a dose-response model (Rose et al., 1991; DuPont et al., 1995; Haas et al., 1996).
There is no corresponding research applying these same techniques to *G. lamblia* and *C. parvum* in oysters.

Exposure assessment is the public health perspective of the model (DiNovi, 2000). It takes into account the number and size of the oysters per meal, the potential presence of the pathogen in those oysters, as well as the susceptibility of the individual to infection. Previous studies are thought to have underestimated the concentration of *G. lamblia* and *C. parvum* in the environment due to methodology limitations (Gibson et al., 1998).

A risk characterization for *G. lamblia* and *C. parvum* in oysters can now be developed. This is a mathematical model incorporating all previous information to determine overall risk to the public (DiNovi, 2000). The risk characterization model includes information such as the number of oysters consumed/meal, the weight of oysters consumed/meal, the pathogen distribution in the oysters, the infectious dose of the pathogens and the number of oyster meals over a particular time period.

Acceptable risks have been established for these organisms in potable water supplies. An acceptable low level risk for treatment plants using source water is thought to be an average concentration of fewer than 2 oocysts/100 liter for *Cryptosporidium* (Haas et al., 1996) and fewer than 0.7 cysts/100 liter for *Giardia* (Regli et al., 1991).

Waterborne protozoan pathogens have raised significant public and scientific interest. For the purpose of developing risk models for these pathogens in oysters, however, more data and more sensitive techniques are needed since current data underestimate the number of cases (Gibson et al., 1998). Future research needs to focus on exposure assessment. This is vital, since reducing the exposure reduces the risk involved (Gibson et al., 1998).
METHODOLOGY

Research Goals

Based on the numerous potential sources of contamination to the Great Bay Estuarine System and the proximity of those sources to the oyster beds, the research hypothesis was two-fold. It was hypothesized that *G. lamblia* and/or *C. parvum* would be detected in oysters harvested from Great Bay and that the multiplex PCR/nested PCR analysis method would provide the specificity and sensitivity required for the development of a monitoring program.

Determination of Cyst/Oocyst Concentration

Determination of *G. lamblia* cyst (Waterborne Technologies, New Orleans, LA, gerbil, H3 isolate, live) and *C. parvum* oocyst (Waterborne Technologies, New Orleans, LA, calf, Iowa isolate, live) concentrations was done using hemocytometer (Hausser) counts. Three 10-fold dilutions were done on separate 1-ml aliquots of cysts and oocysts. A 100-μl pipet tip was used to transfer approximately 10-μl of the $10^{-3}$ *G. lamblia* cyst or *C. parvum* oocyst dilution to both chambers of the hemocytometer. Cysts/oocysts were counted in the 1 mm center square and the four 1 mm corner squares. The concentration of *G. lamblia* cysts was determined to be $2.3 \times 10^6$ /ml and the concentration of *C. parvum* oocysts was calculated to be $1.2 \times 10^6$/ml.
DNA Purification

Two individual deionized water samples (1-ml) were seeded with $10^6$ live *G. lamblia* cysts and $10^6$ live *C. parvum* oocysts. The two seeded deionized water samples were alternately frozen (-80° C) and thawed (57° C) for 3 cycles, then heated to 95° C for 5 minutes and immediately put on ice. The liberated DNA was mixed with phenol:chloroform:isoamyl alcohol (25:24:1), vortexed for 1 minute and centrifuged at 10,000 x $g$ for 10 minutes. The aqueous phase was then extracted using a 200-μl pipet, placed in a sterile 1.5-ml microcentrifuge tube and an equal volume of chloroform:isoamyl alcohol (25:1) was added. The mixture was vortexed for 1 minute and centrifuged at 10,000 x $g$ for 10 minutes. The upper aqueous layer was removed using a 200-μl pipet, placed in a sterile 1.5-ml microcentrifuge tube and two volumes of ice cold 95% ethanol were added. The DNA was allowed to precipitate at -20° C for 2 hours then centrifuged at 12,000 x $g$ for 20 minutes. The 95% ethanol supernatant was removed and the pelleted DNA was washed in 400-μl of 70% ethanol. After centrifugation at 12,000 x $g$ for 5 minutes, the 70% ethanol supernatant was removed and the pellet vacuum dried under medium heat. The DNA pellet was resuspended in 100-μl RNase/ DNase free water and analyzed for potential protein contamination and DNA content using a spectrophotometer (Shimadzu UV 2401-PC). Pure preparations of DNA have an OD$_{260}$/OD$_{280}$ value of 1.8 (Sambrook et al., 1989). The sample of *G. lamblia* DNA was determined to be relatively pure, with a spectrophotometer reading (OD$_{260}$/OD$_{280}$) of 1.8. Spectrophotometer readings for *C. parvum* (OD$_{260}$/OD$_{280}$) showed minimal protein contamination with a reading at 1.6. *G. lamblia* DNA content was assessed to be
6.40 ng/ml. The DNA content of Cryptosporidium was similarly analyzed, with a resulting concentration of 3.13 μg/ml.

**Seeded Water Samples**

At the start of this work, separate 1-ml aliquots of deionized water were seeded with $10^6$ C. parvum oocysts and $10^6$ G. lamblia cysts to determine operational parameters for PCR analysis. Following observation of predicted banding patterns in PCR analysis (211-bp for G. lamblia and 600-bp for C. parvum, as determined by the Primer Select software), subsequent seeded water samples (1-ml) were analyzed via PCR using reduced concentrations of G. lamblia cysts ($10^3$) and C. parvum oocysts ($10^3$).

**Seeded Oyster Extract Samples**

After PCR analysis of seeded water samples, aliquots (10-ml) of previously frozen oyster extract (Crassostrea virginica) were seeded with $10^6$ G. lamblia cysts and $10^6$ C. parvum oocysts to determine PCR parameters using the oyster extract as a substrate. Following the observation of predicted banding patterns in PCR analysis (211-bp for G. lamblia and 600-bp for C. parvum, as determined by the Primer Select software), the concentration of G. lamblia cysts and C. parvum oocysts was reduced to $10^3$.

**Oyster Sample Collection**

Three sites were sampled in the Great Bay Estuary; Nannie's Island, Adam's Point and the Oyster River (Figure 3). Salinity and water temperature were noted at each
sampling site. The sample size was 25 oysters per site. Oyster samples from each site were contained in separate collection bags and stored at 4°C. The oyster samples were processed within 24 hours of collection.

Each oyster was shucked and the entire oyster shell contents, including any extract, were placed in a sterile blender (Waring). Oyster samples used as positive controls were seeded with $10^3$ G. lamblia cysts and $10^3$ C. parvum to determine if the oyster meat itself would inhibit the PCR process or if environmental inhibitors, such as humic acid, were present. The oyster samples were homogenized at high speed for 3 minutes. The homogenized oyster sample was weighed and then filtered through a 50-mesh sterile sieve (Fisher) into a 125-ml Erlenmeyer flask. Aliquots containing 25-ml of the oyster sample were placed into 50-ml tubes.
FIGURE 3. Sampling Sites in the Great Bay Estuarine System, New Hampshire

(Adapted from Nelson, 1982)
Ethyl Acetate Sedimentation

To reduce the bulk of the oyster sample, a sedimentation procedure was utilized. Approximately 14-ml of sterile water was added to the strained oyster sample along with 8-ml ethyl acetate. As an organic solvent, ethyl acetate can dissolve the oyster cell membrane lipids, rupturing the cells and allowing access to the contents. Also, high concentrations of ethyl acetate denature or unfold other proteins that are then degraded. The sample was shaken vigorously for 30 seconds and then centrifuged at 500 x g for 10 minutes. Four layers resulted, which consisted of ethyl acetate, oyster tissue, water and the pellet. The upper layers were removed by dislodging the tissue layer, using a 10-µl pipet tip, and decanting the water and ethyl acetate, leaving the pelleted protozoa and any shell or sediment debris. The pellet was resuspended in 10-ml of sterile water.

Immunomagnetic Separation

At the start of this work, biotinylated primers for *G. lamblia* and *C. parvum* were designed to be used in conjunction with streptavidin-coated paramagnetic beads (Dynal, Lake Success, N.Y.) using an adaptation of a procedure developed by Regan and Margolin (1997). The paramagnetic beads were washed before use in 200-µl wash buffer (5 mM Tris-HCl, pH 8.0 and 1.0 M NaCl). Each primer was modified with the addition of a biotin molecule at the 5’end and a spacer arm was added to prevent any binding hindrance (Integrated DNA Technologies, Coralville, IA). A water sample (1-ml) seeded with purified *G. lamblia* DNA (6.2 µg/ml) and *C. parvum* DNA (3.1 µg/ml) was placed in a 1.5-ml microcentrifuge tube along with 1-µl of each biotinylated primer. Ten to 50-µl of streptavidin-coated paramagnetic beads (200 µg) were added to the seeded...
water sample and incubated, with rotation (20 rpm), at room temperature for 30 minutes. The paramagnetic beads were concentrated using a magnetic particle concentrator rack (Dynal, Lake Success, NY) and the supernatant was removed. The beads were washed three times in 200-µl wash buffer (1.0 M NaCl and 5 mM Tris-HCl, pH 8.0). The *G. lamblia* and *C. parvum* nucleic acids were then resuspended in 200-µl 1X PBS.

This technique was problematic when testing the oyster samples as exogenous biotin in the oyster meat bound up available streptavidin sites on the beads. This prevented the biotinylated primers from adhering to the magnetic beads. The protocol was revised to prevent this problem.

Immunomagnetic separation was utilized to prevent inhibition of molecular techniques. The Dynabeads GC-Combo reagents (Dynal, Lake Success, NY) were equilibrated to room temperature and a 1:10 dilution of 10X SL-Buffer A was prepared. A 10-ml water sample was transferred to a 15-ml flat back glass tube (Dynal, Lake Success, N.Y.) for immunomagnetic separation and 1-ml of 10X SL-Buffer B was added. Both the anti-*Giardia* and anti-*Cryptosporidium* dynabeads were vortexed and 100-µl of each suspension was added to the sample. The mixture was rotated (20 rpm) at room temperature for one hour to ensure optimum binding. Following the one-hour incubation, the flat back tube was placed in a magnetic particle concentrator rack (Dynal, Lake Success, N.Y.) and rotated (20 rpm) for 30 seconds at room temperature. The resulting pellet was resuspended in 1ml of prepared 1X SL-Buffer A and transferred to a sterile 1.5-ml microcentrifuge tube. The suspension was replaced in the magnetic particle concentrator rack and rotated (20 rpm) at room temperature for 30 seconds. The
supernatant was aspirated off using a 200-μl pipet tip and the pellet was resuspended in 200-μl of 1X PBS.

Nucleic Acid Release

The *G. lamblia* and *C. parvum* nucleic acids were released through a series of freeze thaw steps following the immunomagnetic bead capture. The oyster sample (200-μl) in a sterile 1.5-ml microcentrifuge tube was placed at -80°C for approximately 5 minutes and then transferred to a 57°C water bath until thawed, approximately 3 minutes. This process was repeated three times. The sample was then heated to 95°C for 5 minutes and immediately put on ice until further analysis.

Multiplex Polymerase Chain Reaction

Separate deionized water samples (1-ml) containing purified *G. lamblia* DNA (6.2 μg/ml) and *C. parvum* DNA (3.1 μg/ml) were analyzed using separate PCR assays. The multiplex procedure was developed and optimized using purified *G. lamblia* DNA (6.2 μg/ml) and *C. parvum* DNA (3.1 μg/ml) in seeded water samples (1-ml).

An adaptation of the PCR procedure cited by Rochelle et al. (1997a) was used for the multiplex detection of *G. lamblia* and *C. parvum* DNA. At the start of this work, it was noted that the *C. parvum* DNA was amplified much less efficiently than *G. lamblia*. The weak amplification of *C. parvum* was evident even with an increase in annealing time (from 54°C for 30 seconds to 54°C for 1 minute) and extension time from 72°C for 30 seconds to 72°C for 45 seconds. An adjustment of the *C. parvum* upstream and downstream primer concentrations (1.0 μM to 1.5 μM) along with an increase in Taq
polymerase concentration to 2.5 Units resulted in stronger product amplification. In
order to reduce the steps involved in optimizing the multiplex technique, each primer
used in the multiplex assay was modified by the addition of a 20-mer universal primer
sequence to the 5' end (Shuber et al., 1995). The following components were added to
the multiplex reaction: 47.5-μl RNase/DNase free water, 10-μl sample, 12-μl (25 mM)
MgCl2, 15-μl 10 X PCR buffer (Perkin Elmer), 2.5-μl (10 mM) of each dNTP, 1.5 μM of
each C. parvum primer (Table 2) and 1.0 μM of each G. lamblia primer (Table 2) for a
final reaction volume of 100-μl.

Following a hot start at 94°C for 5 minutes, 2.5 U AmpliTaq DNA polymerase
were added to each tube. Cycling conditions (Perkin Elmer 2400 Thermocycler) were 40
cycles at 94°C for 30 seconds, 54°C for 1 minute, 72°C for 45 seconds, and a final
extension step of 72°C for 5 minutes. The samples were stored at 4°C until further
analysis.
**TABLE 2.** Multiplex PCR Primers for *C. parvum* and *G. lamblia*

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. parvum</em> - upstream</td>
<td>5'GCG GTC CCA AAA GGG TCA GTA GCA ATC CTC TGC CGT ACA GG 3'</td>
</tr>
<tr>
<td><em>C. parvum</em> - downstream</td>
<td>5' GCG GTC CCA AAA GGG TCA GTG TTG CTC CAT TAT CAC TCG GTT TA 3'</td>
</tr>
<tr>
<td><em>G. lamblia</em> - upstream</td>
<td>5' GCG GTC CCA AAA GGG TCA GTG TAG TGC TCA ACG AGC AGC T 3'</td>
</tr>
<tr>
<td><em>G. lamblia</em> - downstream</td>
<td>5' GCG GTC CCA AAA GGG TCA GTT TAG TGC TTT GTG ACC ATC GA 3'</td>
</tr>
</tbody>
</table>
Nested PCR

The following components were added to the nested reaction in 0.2-ml thin-walled microcentrifuge tubes: 48.5-μl RNase/DNase free water, 10-μl previous PCR reaction, 12-μl (25mM) MgCl₂, 15-μl 10X PCR buffer (Perkin Elmer), 2.5-μl (10mM) of each dNTP, 1.0 μM of each *C. parvum* nested primer (Table 3) and 1.0 μM of each *G. lamblia* nested primer (Table 3), for a final reaction volume of 100-μl.

Following a hot start at 94° C for 5 minutes, 2 U AmpliTaq DNA polymerase were added to each tube. Cycling conditions (Perkin Elmer 2400 Thermocycler) were 40 cycles at 94° C for 30 seconds, 57° C for 1 minute, 72° C for 45 seconds, and a final extension step of 72° C for 5 minutes. The samples were stored at 4° C until further analysis.
### TABLE 3. Nested PCR Primers for *C. parvum* and *G. lamblia*

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. parvum</em> - upstream</td>
<td>5' GTT GCT CCA TTA TCA CTC GGT TTA 3'</td>
</tr>
<tr>
<td><em>C. parvum</em> - downstream</td>
<td>5' CTT GTG TCT TCT TIG CTG GGA TAG 3'</td>
</tr>
<tr>
<td><em>G. lamblia</em> - upstream</td>
<td>5' GAC CGC GAG AAG GCC GAA CG 3'</td>
</tr>
<tr>
<td><em>G. lamblia</em> - downstream</td>
<td>5' GCC CGC CCT GGA TCT TCG AGA 3'</td>
</tr>
</tbody>
</table>
Agarose Gel Electrophoresis

The PCR products were separated and visualized on 2% low EEO agarose gels made in 1X tris-borate-EDTA buffer, containing 0.5 μg/ml ethidium bromide and run at 70 volts per minute. The PCR products were compared to a 100-base-pair ladder (Gibco-BRL) with a positive result being the visualization of a 211-base-pair band (G. lamblia) band and a 600-base-pair band (C. parvum).

The nested PCR products were visualized using 2% low EEO agarose gels made in 1X tris-borate-EDTA buffer, containing 0.5 μg/ml ethidium bromide and run at 70 volts per minute. The nested PCR products were compared to a 25-base-pair ladder (Gibco-BRL). A positive nested result was the appearance of an 87-base-pair product (G. lamblia) and a 97-base-pair product (C. parvum).

Fluorescent Microscopy

Oyster samples were analyzed microscopically (Olympus UV microscope) for confirmation of the presence of Giardia species and Cryptosporidium species using the MeriFluor Cryptosporidium/Giardia Direct Immunofluorescent Detection Procedure. The Dynabeads were dissociated from the 100-μl oyster sample using 20-μl 1N HCl and vortexing for 30 seconds. The sample was incubated at room temperature for 10 minutes and then vortexed for 20 seconds. The sample (10-μl), a positive control (10-μl), and a negative control (10-μl) were pipeted onto separate wells of a slide and allowed to air dry for approximately 30 minutes. The positive control consisted of a formalized stool preparation of Cryptosporidium oocysts and Giardia cysts and the negative control was formalized stool without oocysts and cysts. One drop of the detection reagent (FITC-
labeled anti-\textit{Giardia} and anti-\textit{Cryptosporidium} monoclonal antibodies) and one drop of the counterstain (Eriochrome Black solution) were placed in each well. The slide was incubated for 30 minutes in a humidified chamber at room temperature, then gently washed with 1X wash buffer. One drop of mounting medium was added to each well and a coverslip (22 x 50 mm) was applied. The sample was analyzed microscopically at 40 x magnification for the presence of \textit{Giardia} species and \textit{Cryptosporidium} species (Olympus UV microscope). \textit{Giardia} species cysts and \textit{Cryptosporidium} species oocysts fluoresce bright green and were distinguished based on morphological characteristics. \textit{Cryptosporidium} oocysts are round, 2-6 \textmu m in size and often have a visible suture line while \textit{Giardia} cysts are oval shaped and 8-12 \textmu m in size.

\textbf{Quality Control}

To reduce the possibility of sample contamination, there were several precautions applied to each part of the process, including the use of autoclaved and UV-treated microcentrifuge tubes, UV-treated water, aerosol-resistant pipet tips and ethanol-treated work areas.

Two samples of oysters (242 g) were purchased from a local supermarket, source of oysters unknown, to test as a control sample. One oyster control sample (121 g) was analyzed using the multiplex PCR and nested PCR assays and MeriFluor \textit{Cryptosporidium/Giardia} Direct Immunofluorescent Detection. The second oyster control sample (121 g) was seeded with $10^3$ cysts and $10^3$ oocysts to determine if the oyster meat itself was inhibitory to the PCR assays.
PCR controls for each assay included negative controls of *G. lamblia* primers (1.0 μM) with *C. parvum* DNA and *C. parvum* primers (1.0 μM) with *G. lamblia* DNA. Positive controls used in the assays included oyster meat seeded with *G. lamblia* cysts (10^3) and *C. parvum* oocysts (10^3). Purified *G. lamblia* (6.4 μg/ml) and *C. parvum* DNA (3.1 μg/ml) were used as positive controls.

**Efficiency of Technique**

In order to determine the efficiency of the detection procedure, each step of the process was analyzed. A 10-ml sample of deionized water was seeded with 10^3 *G. lamblia* cysts and 10^3 *C. parvum* oocysts. Sample loss was determined at each point of the procedure by removing an aliquot (100-μl), diluting 1:10 and directly counting the *G. lamblia* cysts and *C. parvum* oocysts (Figure 4). The seeded oyster sample, at the point of PCR, was diluted to extinction using 10-fold dilutions.
FIGURE 4. Recovery Efficiency of *G. lamblia* Cysts and *C. parvum* Oocysts from Deionized Water

Seed 10-ml deionized water sample

- *C. parvum* oocysts: $5.0 \times 10^3$
- *G. lamblia* cysts: $4.3 \times 10^3$

↓

Vortex 30 seconds

↓

Filter through 50-mesh sieve

↓

Direct counts

- *C. parvum* oocysts: $5.0 \times 10^3$ (100% recovery)
- *G. lamblia* cysts: $4.2 \times 10^3$ (98% recovery)

↓

Ethyl acetate sedimentation

↓

Direct counts

- *C. parvum* oocysts: $4.3 \times 10^3$ (86% recovery)
- *G. lamblia* cysts: $3.8 \times 10^3$ (90% recovery)

↓

Immunomagnetic separation

↓

Direct counts

- *C. parvum* oocysts: $3.2 \times 10^3$ (74% recovery)
- *G. lamblia* cysts: $2.5 \times 10^3$ (66% recovery)

↓

Overall Efficiency of Process

- *C. parvum* oocysts: $3.2 \times 10^3$ (64% recovery)
- *G. lamblia* cysts: $2.5 \times 10^3$ (58% recovery)
RESULTS

Purified *G. lamblia* and *C. parvum* DNA

Figures 5 and 6 show the agarose gel electrophoresis results of *G. lamblia* and *C. parvum*, respectively, utilizing purified DNA. Briefly, two individual water samples (1ml) were seeded with $10^6$ *G. lamblia* cysts and $10^6$ *C. parvum* oocysts. The nucleic acids were released using the heat shock technique, purified with phenol:chloroform:isoamyl alcohol (25:24:1), and vacuum dried. Potential protein contamination was assessed for each organism using spectrophotometric (Shimadzu UV 2401-PC) readings at OD$_{260}$/OD$_{280}$. Minimal protein contamination was noted for *G. lamblia* at a reading of 1.8 and *C. parvum*, with a reading at 1.6. The DNA yields were determined using a spectrophotometer (Shimadzu UV 2401-PC) and assessed to be 6.4 μg/ml for *G. lamblia* and 3.1 μg/ml for *C. parvum*.

The subsequent PCR analysis of the individual samples showed the strong appearance of amplicons of predicted size. These were determined by Primer Select software, for each organism, indicating proper primer annealing and suitable PCR operational parameters. These amplicons (211-bp for *G. lamblia* and 600-bp for *C. parvum* (Figures 5 and 6, lane 2)) were compared to a 100-bp standard that was run simultaneously (Figures 5 and 6, lane 1). A control reaction utilizing lambda DNA (Figures 5 and 6, lane 3) indicated the PCR assay was working properly while the absence of amplicons in the negative control lanes (Figure 5 and 6, lane 4) indicated that the primers were not producing false positives due to cross reaction. Excess bands, evident in both gels (Figures 5 and 6, lane 2), were thought to be the result of primer dimer formation and/or nonspecific binding.
FIGURE S. Agarose gel analysis demonstrating detection of *G. lamblia* DNA (6.1 μg/ml) in a water sample (1-ml). Lanes: 1, 100-bp standard; 2, 211-bp product (arrow); 3, lambda positive control; 4, negative control.
FIGURE 6. Agarose gel analysis demonstrating detection of *C. parvum* DNA (3.1 μg/ml) in a water sample (1-ml). Lanes: 1, 100-bp standard; 2, 600-bp product (arrow); 3, lambda positive control; 4, negative control.
Multiplex PCR

Figure 7 shows the development of the multiplex PCR assay, demonstrated using water samples (1 ml) seeded with 10-μl purified *G. lamblia* DNA (6.1 μg/ml) and 10-μl *C. parvum* DNA (3.1 μg/ml). Modifications to the *G. lamblia* upstream and downstream primers and *C. parvum* upstream and downstream primers included the addition of a 20-mer universal primer sequence to the 5' end. The addition of the universal primer sequence minimized the many steps, including adjustments to the MgCl₂, dNTP, and primer concentrations, required to optimize the multiplex protocol. The predicted product sizes, as determined by Primer Select software, were 211 bp for *G. lamblia* and 600 bp for *C. parvum*. A comparison of amplicons for *G. lamblia* and *C. parvum* produced in the assay to a 100 bp ladder indicated positive multiplex amplification (Figure 7, lane 2).
FIGURE 7. Agarose gel analysis demonstrating multiplex detection of *C. parvum* DNA (3.1 µg/ml) and *G. lamblia* DNA (6.1 µg/ml) in a water sample (1-ml). Lanes: 1, 100-bp standard; 2, 211-bp product (arrow B) and 600-bp product (arrow A); 3, lambda positive control; 4, negative control.
Nested PCR

Increased specificity and sensitivity was provided by the inclusion of nested PCR into the detection protocol. The nested reaction confirmed the results observed in the multiplex assay by the appearance of strong amplicons; 87-bp *G. lamblia* (Figure 8, lane 2) and 97-bp *C. parvum* (Figure 9, lane 2), as predicted by the Primer Select software. Negative control lanes lacked any product, indicating that the *G. lamblia* nested primers were not cross binding with the *C. parvum* DNA (Figure 8, lane 4) and *C. parvum* primers were not binding with *G. lamblia* DNA (Figure 9, lane 4). Internal primers that were designed for the nested reaction lacked the universal primer sequence as nested analysis was done individually, rather than in a multiplex design.

Excess bands that were evident in the multiplex analysis were absent in the nested reaction, thereby reducing the potential for reporting false positives.
FIGURE 8. Agarose gel analysis demonstrating nested detection of *G. lamblia*. Lanes: 1, 25-bp standard; 2, 87-bp product (arrow); 3, lambda positive control; 4, negative control.

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FIGURE 9. Agarose gel analysis demonstrating nested PCR detection of *C. parvum*. Lanes: 1, 25-bp standard; 2, 97-bp product (arrow); 3, lambda positive control; 4, negative control.
**G. lamblia** Cysts and **C. parvum** Oocysts

**PCR analysis**

Figure 10 shows the agarose gel electrophoresis of **G. lamblia** cysts (lane 2) and **C. parvum** oocysts (lane 4), previously analyzed via PCR. Individually seeded water samples (1ml) consisting of $10^3$ **G. lamblia** cysts and $10^3$ **C. parvum** oocysts were subjected to the heat shock procedure to liberate the nucleic acid. Without further purification, the liberated DNA was detected using PCR. Positive results, indicating that the heat shock technique was sufficient to liberate the nucleic acid for analysis, were indicated by the observation of the 211 bp **G. lamblia** amplicon and the 600 bp **C. parvum** amplicon, as predicted by the Primer Select software (Figure 10, lanes 2 and 4). Two positive controls were included in the PCR assay, consisting of purified **G. lamblia** DNA and purified **C. parvum** DNA. Excess bands were noted in all lanes, with the exception of the negative controls (lane 6). This may indicate that the excess amplicons are the result of cross binding rather than primer dimer formation, showing the necessity of the additional nested PCR step to minimize false positive results.
FIGURE 10. Agarose gel analysis demonstrating PCR detection of *C. parvum* oocysts \((10^6)\) and *G. lamblia* cysts \((10^3)\) in water. Lanes: 1, 100-bp standard; 2, 211-bp product (arrow B); 3, *G. lamblia* DNA positive control; 4, 600-bp product (arrow A); 5, *C. parvum* DNA positive control; 6, negative control.
Multiplex PCR

Figure 11 shows the detection of *G. lamblia* cysts and *C. parvum* oocysts via multiplex PCR. The presence of the predicted amplicons for each organism, as determined by Primer Select Software, is evidence that the multiplex parameters are sufficient for amplification of the organisms, including heat shock as a means of nucleic acid release. In order to increase the amplification of the *C. parvum* amplicon, it was necessary to increase the primer concentration from 1.0 μm to 1.5 μm. Previous to this increase in primer concentration, a lack of reproducibility for *C. parvum* was observed. As this was not problematic when analyzing the purified *C. parvum* DNA, it was thought that the heat shock technique was producing this effect due to the presence of excess protein that was preventing optimal annealing.
FIGURE 11. Agarose gel analysis demonstrating multiplex PCR detection of *C. parvum* oocysts (10^3) and *G. lamblia* cysts (10^3) in water (1-ml). Lanes: 1, 100-bp standard; 2, 211-bp product (arrow B) and 600-bp product (arrow A); 3, *G. lamblia* cyst positive control; 4, *C. parvum* oocyst positive control; 5, negative control.
Nested PCR Detection

The nested reaction (Figures 12 and 13) confirmed the results observed in the multiplex assay with the production of predicted amplicons 87-bp for *G. lamblia* and 97-bp for *C. parvum*. The utilization of nested primers increased the specificity and sensitivity of the protocol by ensuring that the bands produced in the multiplex assay were not false positive results. A positive control was included which consisted of a PCR product from *G. lamblia* cysts and *C. parvum* oocysts.
FIGURE 12. Agarose gel analysis demonstrating nested PCR detection of *G. lamblia* cysts in water. Lanes: 1, 25-bp standard; 2, 87-bp product (arrow); 3, *G. lamblia* positive control; 4, lambda positive control; 5, negative control.
FIGURE 13. Agarose gel analysis demonstrating nested PCR detection of *C. parvum* oocysts in water. Lanes: 1, 25-bp standard; 2, 97-bp product (arrow); 3, *C. parvum* positive control; 4, negative control.
G. lamblia Cysts and C. parvum Oocysts in Oyster Extract

Oyster extract (10 ml) was initially tested for potential inhibition of the PCR assay by the oyster meat or environmental inhibitors. Prior seedings with $10^6$ G. lamblia cysts and $10^6$ C. parvum oocysts in individual aliquots of oyster extract sample (10 ml) showed no product, demonstrating that inhibitory factors were present in the shellfish extract. The use of biotinylated primers and streptavidin paramagnetic beads was initially used to draw the liberated G. lamblia DNA and C. parvum DNA from the oyster sample. This technique was ineffective when testing oyster extract due to the presence of exogenous biotin, which was binding up available sites on the streptavidin beads, preventing the biotinylated G. lamblia and C. parvum primers from binding to the beads.

The use of the immunomagnetic separation technique allowed for easy removal and concentration of G. lamblia cysts and C. parvum oocysts from the oyster extract. PCR analysis used in conjunction with the immunomagnetic separation technique produced positive results with the observation of the predicted product sizes, 211 bp G. lamblia product and 600 bp C. parvum product. Seedings of G. lamblia cysts and C. parvum oocysts into oyster extract (10 ml) were reduced to $10^3$ cysts and $10^3$ oocysts for subsequent PCR assays (Figures 14 and 15) and multiplex PCR detection (Figure 16). The presence of strong amplicons indicated that the immunomagnetic separation was effective in withdrawing the cysts and oocysts from the oyster homogenate and removing inhibitors. Prior attempts to detect G. lamblia and C. parvum without the use of immunomagnetic separation produced no product.

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FIGURE 14. Agarose gel analysis demonstrating PCR detection of *G. lamblia* cysts (10^3) in oyster extract (10-ml). Lanes: 1, 100-bp standard; 2, *G. lamblia* positive control; 3, 211-bp product (arrow); 4, negative control; 5, negative control; 6, multiplex positive control.
FIGURE 15. Agarose gel analysis demonstrating PCR detection of *C. parvum* oocysts ($10^3$) in oyster extract (10-ml). Lanes: 1, 100-bp standard; 2, empty; 3, empty; 4, 600-bp product (arrow); 5, negative control; 6, *C. parvum* positive control; 7, multiplex positive control.
FIGURE 16. Agarose gel analysis demonstrating multiplex PCR detection of *C. parvum* oocysts (10³) and *G. lamblia* cysts (10³) in oyster extract. Lanes: 1, 100-bp standard; 2, *G. lamblia* positive control; 3, *C. parvum* positive control; 4, multiplex positive control; 5, 211-bp product (arrow B) and 600-bp product (arrow A); 6, negative control.
**G. lamblia** Cysts and *C. parvum* Oocysts in Oyster Meat

**Multiplex PCR**

Figure 17 shows multiplex analysis of *G. lamblia* cysts and *C. parvum* oocysts in previously frozen (-80°C) oyster meat samples (120 g). Briefly, the oyster meat samples were seeded with *G. lamblia* cysts (10^3) and *C. parvum* oocysts (10^3), processed using the ethyl acetate sedimentation technique and immunomagnetic separation to concentrate the protozoa and remove potential inhibitors. The nucleic acids were released via heat shock and analyzed using multiplex PCR. A comparison of product sizes for *G. lamblia* (211-bp) and *C. parvum* (600-bp) produced in the assay to a 100-bp ladder indicated positive multiplex analysis (Figure 17, lane 2). Although excess bands in the multiplex assay were noted, they were considered to be extraneous since they were not verified by nested PCR. The interpretation of the multiplex analysis was not compromised as the miscellaneous bands were of incorrect size and not apparent following the subsequent nested PCR analysis.

The appearance of bands of predicted size, as determined by Primer Select Software, for both *G. lamblia* and *C. parvum* indicated that combining the ethyl acetate sedimentation technique with the immunomagnetic separation technique was sufficient to remove inhibitors to subsequent molecular analysis. Previous work that attempted to analyze the oyster meat sample without utilizing the ethyl acetate sedimentation and/or the immunomagnetic separation method proved useless, as amplicons were not evident in the gel electrophoresis.
FIGURE 17. Agarose gel analysis demonstrating multiplex PCR detection of *C. parvum* oocysts (10^3) and *G. lamblia* cysts (10^3) in oyster meat. Lanes: 1, 100-bp standard; 2, 211-bp product (arrow B) and 600-bp product (arrow A); 3, multiplex positive control; 4, *G. lamblia* positive control; 5, *C. parvum* positive control; 6, negative control; 7, negative control.
Environmental Oyster Samples

Multiplex PCR Detection of *G. lamblia* Cysts and *C. parvum* Oocysts

In order to determine the applicability of the detection methods developed in the laboratory to actual field samples, twenty-five oysters were collected from approved and prohibited sites in the Great Bay Estuary, kept at 4° C during collection and processed in the laboratory as previously mentioned. Multiplex analysis of the environmental oyster samples produced positive results as evident by the predicted amplicons, as determined by Primer Select software; 211-bp for *G. lamblia* and 600-bp for *C. parvum* (Figure 18, lane 4). Excess bands were apparent in the multiplex assay but were not observed in the nested assay, suggesting that the bands were the result of primer dimer formation or nonspecific binding. Positive controls were included in this assay. Each positive control consisted of oyster meat (10 ml) seeded with *G. lamblia* cysts (10^3) (lane 7) and *C. parvum* oocysts (10^3) (lane 8), which had been processed simultaneous to the environmental samples. Amplicon formation was not apparent in the negative control lane (lane 9).
FIGURE 18. Agarose gel analysis demonstrating multiplex PCR detection of *G. lambia* cysts and *C. parvum* oocysts in environmental oyster samples. Lanes: 1, 100-bp standard; 2, negative control; 3, empty; 4, 211-bp product (arrow B) and 600-bp product (arrow A); 5, empty; 6, multiplex positive control; 7, *G. lambia* positive control; 8, *C. parvum* positive control; 9, negative control.
Nested PCR

Nested PCR assays (Figures 19 and 20) were used to confirm the multiplex results by utilizing internal primers specific for target sequences on the PCR amplicon. The nested PCR products were separated and visualized on agarose gels with the appearance of predicted amplicon sizes evident for *G. lamblia* (87-bp) (Figure 19, lane 3) and *C. parvum* (97-bp) (Figure 20, lane 3). Positive controls included in the assay consisted of PCR product from *G. lamblia* cysts (10^3) and *C. parvum* oocysts (10^3).
FIGURE 19. Agarose gel analysis demonstrating nested PCR detection of *G. lamblia* cysts in environmental oyster samples. Lanes: 1, 25-bp standard; 2, *G. lamblia* positive control; 3, 87-bp product (arrow); 4, negative control.
FIGURE 20. Agarose gel analysis demonstrating nested PCR detection of *C. parvum* oocysts in environmental oyster samples. Lanes: 1, 25-bp standard; 2, *C. parvum* positive control; 3, 97-bp product (arrow); 4, negative control.
Analysis of Oyster Control Samples

Multiplex PCR

Figure 21 shows the agarose gel electrophoresis for the oyster control samples. Briefly, two control samples of oysters (242 g) were purchased from a local supermarket, original source of oysters unknown. One oyster control sample (121 g) was tested for the presence of *G. lamblia* cysts and *C. parvum* oocysts and the second control (121 g) was seeded with $10^3$ *G. lamblia* cysts and $10^3$ *C. parvum* oocysts and analyzed to observe for any potential inhibition caused by the oyster meat itself. Both control samples were processed simultaneously utilizing the ethyl acetate sedimentation procedure, the immunomagnetic separation technique and multiplex PCR. A positive result for the presence of *G. lamblia* and *C. parvum* would be the observation of the predicted product sizes, as determined by Primer Select software; 211-bp for *G. lamblia* and 600-bp for *C. parvum*. These amplicons were not evident in the unseeded oyster control sample, indicating that *G. lamblia* and *C. parvum* were not present. A comparison of product sizes in the seeded oyster control for *G. lamblia* and *C. parvum* to a 100-bp ladder indicated positive multiplex analysis (Figure 21, lane 4). The presence of the predicted product sizes indicated that inhibitors were not present in the oyster control sample.
FIGURE 21. Agarose gel analysis demonstrating PCR detection of *C. parvum* and/or *G. lamblia* in oyster control sample. Lanes: 1, 100-bp standard; 2, *G. lamblia* positive control; 3, *C. parvum* positive control; 4, seeded control sample; 5, no product; 6, negative control.
Nested PCR

Nested PCR assays were conducted on the PCR products from the oyster control samples to increase the sensitivity and specificity of the reaction and to verify the desired amplification. The nested PCR products were separated and visualized on agarose gels (Figures 22 and 23). The predicted amplicons, as determined by Primer Select software, were 87 bp for *G. lamblia* and 97 bp for *C. parvum*. There was no product produced in the nested reaction for either *G. lamblia* (Figure 23, lane 3) or *C. parvum* (Figure 22, lane 3) in the unseeded control oyster sample. The nested analysis of the oyster control sample that was seeded with $10^3$ *G. lamblia* cysts and $10^3$ *C. parvum* oocysts demonstrated a positive result, as evident by the predicted product sizes (Figures 22 and 23, lane 2).
FIGURE 22. Agarose gel analysis demonstrating nested PCR detection of *C. parvum* in oyster control sample. Lanes: 1, 25-bp standard; 2, *C. parvum* positive control; 3, no product; 4, negative control.
FIGURE 23. Agarose gel analysis demonstrating nested PCR detection of *G. lambia* in oyster control sample. Lanes: 1, 25-bp standard; 2, *G. lambia* positive control; 3, no product; 4, negative control.
Immunofluorescence

Environmental oyster samples were analyzed microscopically using the MeriFluor Cryptosporidium/Giardia Direct Immunofluorescent Detection Procedure, following sedimentation and immunomagnetic capture, to detect the presence of Giardia species and/or Cryptosporidium species. Giardia species cysts and Cryptosporidium species oocysts fluoresce bright green and were distinguished based on morphological characteristics. Cryptosporidium oocysts are round, 2-6 μm in size and often have a visible suture line while Giardia cysts are oval shaped and 8-12 μm in size. Both Cryptosporidium oocysts (Figure 24) and Giardia cysts (Figure 25) were observed in the environmental samples corresponding with the results observed using molecular analysis.
FIGURE 24 confirms the molecular results by microscopically determining the presence of *Cryptosporidium* species oocysts (B) in an environmental oyster sample using direct immunofluorescence.
FIGURE 25 confirms the molecular results by microscopically determining the presence of *Giardia* species cysts (A) and *Cryptosporidium* species oocysts (B) in an environmental oyster sample using direct immunofluorescence.
TABLE 4. Detection of *G. lamblia* cysts and *C. parvum* oocysts from Oysters Collected in the Great Bay Estuary using Multiplex PCR, Nested PCR and IFA.

<table>
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<th>DATE</th>
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<th>OYSTER MEAT AMT (g)</th>
<th>MULTIPLEX PCR</th>
<th>NESTED PCR</th>
<th>IFA</th>
<th>Fecal Coliforms*</th>
<th>SALINITY (ppt)</th>
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<td>ND</td>
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<td>30</td>
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<td>neg/pos</td>
<td>neg/pos</td>
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<td>2</td>
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<td>121</td>
<td>neg/neg</td>
<td>neg/neg</td>
<td>neg/neg</td>
<td>NA</td>
<td>NA</td>
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</tbody>
</table>

NA: Not applicable  
ND: Not done  
* indicates a positive result by the observation of predicted amplicons in PCR analysis or cyst/oocyst fluorescence  
* indicates a negative result by the lack of predicted amplicons in PCR analysis or cyst/oocyst fluorescence  
* MPN / 100 ml water
DISCUSSION

Oyster Contamination

Waterborne disease has historically been attributed to viral and bacterial contaminants, yet there have always been incidences of infection resulting from unknown etiologies. The causative agents in some of the periodic outbreaks of non-bacterial and non-viral associated gastroenteritis were recognized to be protozoan pathogens *G. lamblia* and *C. parvum* (CDC, 1996).

*G. lamblia* and *C. parvum* were once classified as freshwater pathogens although their ability to remain infectious in saline environments has recently been observed. These pathogens are transmitted into the environment via the fecal oral route, with infection resulting from the ingestion of contaminated water or contaminated food products, such as oysters. Oysters are filter feeders and can concentrate fecal contaminants from the water so the level of pathogens in the oyster may exceed that seen in the overlying water. Oysters harvested from the Great Bay Estuarine System are suspect as the Estuary is affected by point and nonpoint sources of pollution that are potential sources of *G. lamblia* and *C. parvum.*

A method currently used for the detection of *Giardia* cysts and *Cryptosporidium* oocysts is the immunofluorescence assay, which utilizes microscopy to distinguish between the organisms based on morphology. This method is inadequate when analyzing environmental samples since the antibodies specific for *Giardia* species and *Cryptosporidium* species may antigenically cross-react with algae,
producing false positive results (Rodgers et al., 1995). The auto-fluorescence of some algal cells also increases the potential for researchers to misread the sample. These results may then lead to unwarranted oyster bed closures.

Due to the potential public health risk and the economic importance of shellfish harvesting, it is necessary to develop a method of detection that is rapid and specific. It is improbable that accurate data will consistently result when solely utilizing microscopic evaluation to screen a large number of oyster samples. The time and effort necessary for a large-scale microscopic evaluation of oyster samples is problematic and counterproductive to a high-speed detection system.

The polymerase chain reaction is a rapid technique with a high degree of sensitivity and specificity, although its use with environmental samples is often limited due to molecular inhibitors and small reaction volumes. These limitations can be overcome using an immunomagnetic separation technique prior to PCR analysis. Immunomagnetic separation allows for rapid and selective separation of *Giardia* species and *Cryptosporidium* species from sample concentrates. Since this research does not rely on microscopic evaluation for *G. lamblia* and *C. parvum* identification, the lack of species specificity by the antibodies is not problematic. The utilization of the paramagnetic beads allows for *Giardia* species and *Cryptosporidium* species to be complexed and washed, overcoming potential inhibitors to the PCR assay. The use of immunomagnetic separation also concentrates the original sample, increasing the amount that can be evaluated in the PCR assay. Specificity to the species level is observed during the PCR process.
The multiplex polymerase chain reaction is a technique in which two or more loci are simultaneously amplified in one reaction. This is an ideal technique to use for a monitoring program, as additional pathogens can be added to the detection system, reducing the overall cost and effort involved.

Oyster Sample Processing

In order to conduct analysis of the oyster meat, a technique to reduce the oyster sample bulk was necessary. This initial “de-bulking” step allowed for easier manipulation of the oyster sample and extraction of the parasites.

There are two methods used for the detection of Cryptosporidium oocysts and Giardia cysts in fecal samples, the flotation technique and the ethyl acetate sedimentation technique. The flotation technique utilizes magnesium chloride at a specific gravity of 0.18 in order to float the cysts and oocysts to the surface in the meniscus, allowing for collection (Balows et al., 1991). Disadvantages to using the flotation method include such factors as the inability of ova to concentrate well, the potential for larvae and cysts to collapse and the possibility that operculate eggs may rupture (Balows et al., 1991). This may lead to an underestimation of the actual parasite concentration in the sample. The flotation technique is generally regarded as the least efficient and least sensitive of the two techniques.

An adaptation of the clinical ethyl acetate sedimentation technique was used for this work. It was observed that the addition of ethyl acetate effectively dissolved and displaced oyster tissue, leaving pelleted parasites, along with remaining sediment and shell debris. Centrifugation speeds are crucial, as less than 500 x g would not settle cysts...
and oocysts out of suspension. Centrifugation speeds of 500 x g resulted in consistent recoveries of *Giardia* cysts and *Cryptosporidium* oocysts, as determined by hemocytometer counts.

The ethyl acetate sedimentation technique proved useful in reducing the overall effort involved in the detection of cysts and oocysts in shellfish as the entire oyster is utilized. Previous work had detected cysts and oocysts by looking at portions of the shellfish and/or drawing hemolymph samples (Fayer et al., 1997). There are little data available to determine specific locations where *Giardia* cysts and *Cryptosporidium* oocysts concentrate in the oyster. One study indicates the presence of *Cryptosporidium* oocysts in the hemolymph, intestinal track and on the gills (Fayer et al., 1997). By utilizing a technique that includes the entire oyster in the detection procedure there is less chance of overlooking the presence of pathogens. This would provide a more accurate account of the presence of *G. lamblia* and *C. parvum* in oysters. The sedimentation technique also allowed for a greater sample size to be analyzed, more accurately reflecting the amount people eat. On average, the analysis began with 340 grams of oyster sample and the sedimentation technique reduced it to approximately 170 grams.

Environmental samples contain substances such as humic acids, which are inhibitory to PCR analysis. To overcome environmental inhibitors or inhibition caused by the oyster meat itself, the use of biotinylated primers and streptavidin beads were investigated but found to be ineffective in this work. Initially, it was hypothesized that the binding capabilities of the biotin/streptavidin interaction would be useful in extracting the *G. lamblia* and *C. parvum* nucleic acid from the environmental sample, using an adaptation of the method described by Regan and Margolin (1997). This technique
proved useful in water samples (Regan and Margolin, 1997), but was found to be an inefficient technique for the analysis of oyster samples. This was due to exogenous biotin from the oyster tissue binding available streptavidin sites on the paramagnetic beads, resulting in reduced binding capability of the biotinlyated primers.

Immunomagnetic separation provided a means of extracting the protozoa from the oyster sample without biotin interference. Using antibody-antigen interaction, inhibitors, such as humic acid and the oyster meat itself, were removed through a series of washing steps. Initial multiplex PCR analysis of oyster extract samples and oyster meat samples, without any prior treatment, resulted in little to no product. The incorporation of the immunomagnetic separation technique prior to PCR analysis overcame the inhibitors and concentrated the original sample allowing for concentrations of \( G. \) lamblia cysts and \( C. \) parvum oocysts to be lowered to \( 10^3 \) with predicted banding patterns of 211 bp, \( G. \) lamblia and 600 bp, \( C. \) parvum, evident in PCR analysis (Figure 16, lane 5). The immunomagnetic separation technique allowed for isolation and concentration of \( Giardia \) cysts and \( Cryptosporidium \) oocysts from the oyster sample in less than four hours, with a 66% recovery of cysts and 74% recovery of oocysts (Figure 4).

Following isolation of \( Giardia \) cysts and \( Cryptosporidium \) oocysts, the heat-shock procedure was used for nucleic acid release. The heat-shock procedure allowed the paramagnetic beads to remain attached to the surface of the cysts and oocysts without interference in subsequent PCR analysis. This is in contrast to analysis of shellfish samples using immunofluorescence, as the paramagnetic beads must first be dissociated from the cysts and oocysts. This additional step makes the immunofluorescent assay more labor intensive and increases the potential for sample loss.
Heat shock was an efficient means of nucleic acid release. It was observed microscopically that *G. lamblia* cysts had a tendency to break open more consistently in comparison to the *C. parvum* oocysts. The larger surface area and greater water content of the *Giardia* cysts in comparison to the *Cryptosporidium* oocysts may account for this result. The dense paramagnetic beads naturally settled to the bottom of the tube and a simple centrifuge step (100 x g for 3 minutes) caused the cyst and oocyst shells to also settle out of suspension. *Giardia* and *Cryptosporidium* nucleic acids could be recovered from the supernatant and analyzed for the specific human pathogenic strain using a multiplex polymerase chain reaction.

The development of a multiplex polymerase chain reaction allowed for rapid and sensitive detection of *G. lamblia* and *C. parvum* in oysters. The *G. lamblia* primers targeted the giardin gene, a structural gene that codes for the cytoskeleton. The primers used for *C. parvum* were specific for a heat shock protein. This protein is a 70-kDa protein that is responsible for the organism's heat shock response. By triggering transcription of mRNA and the production of corresponding enzymes, the organism is able to survive increasing temperatures for short durations of time. One study demonstrated that *C. parvum* oocysts remained infectious after incubation at 60°C for 5 minutes (Fayer et al., 1994).

The development of the multiplex assay required several optimization steps. Since multiplex PCR is amplifying multiple loci, there are specific problems associated with development of the assay. These issues include uneven amplification and lack of reproducibility. In order to avoid those problems, reaction parameters such as MgCl₂, dNTP, and primer concentrations had to be optimized. The addition of an universal
primer sequence to the 5' end of each primer reduced the optimization steps needed by stabilizing the kinetics of the multiplex reaction. This 20-mer sequence, taken from the bacteriophage M13mp18, acts to stabilize the reaction kinetics by being incorporated into the newly formed strands during the early rounds of PCR. Therefore, all subsequent rounds of PCR will have the same 20-mer sequence with similar hybridization kinetics (Shuber et al., 1995). By normalizing the kinetics of the assay, primers for different organisms can be utilized without having to evaluate diverse reaction conditions and cycling parameters, reducing the labor involved in multiplex detection.

The G. lamblia and C. parvum primers used in the multiplex procedure were modified by the addition of a universal primer sequence at the 5' end. Multiplex analysis with the universal primer sequence in both seeded water samples and seeded oyster samples all produced amplicons of expected size, 211 bp G. lamblia and 600 bp C. parvum, as predicted by the Primer Select software (Figures 11 and 17). This indicated proper annealing of the primers to target sequences. Excess bands were evident as a result of primer dimers formation or nonspecific binding. The Primer Select software predicted the formation of dimers due to the extensive size of each primer (40-mer). These amplicons were not evident in nested PCR analysis, indicating that they were extraneous bands.

The addition of the universal primer sequence would allow for future studies on shellfish safety to incorporate primers specific for other pathogens such as Vibrio parahaemolyticus, Vibrio vulnificus, and enteric viruses into the multiplex design. This would result in identification of many pathogens in one assay, significantly reducing the effort involved in detection.
One drawback to using a nucleic acid based detection assay such as multiplex PCR, is the inability to determine the viability of an organism. The current method of detection for *G. lamblia* and *C. parvum*, the immunofluorescent assay, also does not distinguish between viable and nonviable organisms. A benefit of the multiplex process, as compared with the immunofluorescent assay, is the simultaneous detection of *G. lamblia* and *C. parvum*. This allows for rapid detection of these pathogens, making the process ideal for a monitoring program. The efficiency of the multiplex technique was determined, using oyster meat samples seeded with $10^3$ *Giardia* cysts and $10^3$ *Cryptosporidium* oocysts and diluting to extinction. It was determined that the multiplex process itself could be sensitive enough to detect 10 cysts/oocysts.

The incorporation of nested PCR into the detection procedure verifies that false positive results have not been generated as a result of nonspecific binding. This process utilized two internal primers that are specific to sequences on the original PCR amplicon. This increased the specificity of the reaction beyond what is determined in a single assay and reduced the potential for false positive results.

Excess banding patterns produced in the multiplex gel were not evident in the nested gel indicating that they were not part of the desired target sequence (Figures 12 and 13). Amplicons of 87-bp for *G. lamblia* and 97-bp for *C. parvum*, as predicted by the Primer Select software, were evident in the gel showing that the target sequences were in fact *G. lamblia* and *C. parvum*.

**Fecal Coliforms as Indicator Organisms**

Classification of shellfish harvesting sites is based on fecal coliform counts using most probable number (MPN) analysis. Sites in the Great Bay are determined to be open
or closed based on the following National Shellfish Sanitation Program guidelines. Approved sites are defined as areas that show a geometric mean of fewer than 14 fecal coliform colonies/100 ml sampled water. Restricted harvesting areas have a geometric mean of fewer than 80 fecal coliforms/100 ml sampled water and prohibited areas have a geometric mean of greater than 80 fecal coliform colonies/100 ml sampled water. These guidelines are based on long-term sanitary surveys involving at least 30 samples (NHEP, 2000).

Samples of the eastern oyster (*Crassostrea virginica*) were taken at specific areas in the Great Bay Estuarine System. Two of the harvesting areas, Nannie’s Island and Adam’s Point, are currently classified as open to harvesting based on most probable number (MPN) analysis of the overlying water. When results of testing are fewer than 14 fecal coliform colonies/100ml water sampled, the area is allowed to remain open to shellfish harvesting (NHEP, 2000). The other site, the Oyster River, is a closed site in which shellfish harvesting is restricted due to fecal contamination. On average, fecal coliform levels in the Oyster River are between 14 and 88 fecal coliforms/100 ml of sampled water, based on MPN analysis (NHEP, 2000).

Fecal coliforms, the indicator organisms for this analysis, are used to indicate the presence or absence of other fecal-borne pathogens; however the use of these organisms as indicators is disputed (Burkhardt et al., 2000). A recent study showed that sunlight could result in up to a 99% reduction in fecal coliform populations (Burkhardt et al., 2000). This may be directly applicable to shallow estuarine environments, such as the Great Bay Estuarine System.
There is also a concern with using fecal coliforms as indicators particularly in areas that discharge treated wastewater. This waste product is treated with chlorine at levels that may render fecal coliform bacteria in river water nonviable. Other pathogens, such as viruses and protozoa, are more resistant to chlorine than fecal coliforms. Thus, any water testing done in that area may show acceptable levels of fecal coliforms and yet the oysters in the area may be exposed to, and concentrating, viral and/or protozoan pathogens. Research has shown that although optimal wastewater treatment, including filtration, can remove greater than 99% of cysts and oocysts, a large number may still be released into the environment and detected post-filtration (42-75% positive) (Rose et al., 1996).

Fecal coliform testing in New Hampshire is performed in both dry weather and following rain events (NHEP, 2000). Although many wastewater treatment plants have been upgraded in the past, they are often not able to handle the excess water from runoff and, as a result, may release improperly treated wastewater into the environment. This wastewater will eventually flow into the Great Bay, contaminating oyster beds. The geometric mean of fecal coliform levels in a three-year study (1993-1996) show much greater concentrations of fecal coliforms following a rain event (NHEP, 2000). The Oyster River, an area sampled for this research, showed geometric means of 26 fecal coliform colonies/100 ml of sampled freshwater (7/100 ml tidal water) in dry weather as compared to an average of 312 fecal coliform colonies/100 ml in freshwater (31/100 ml tidal water) following a rain event (NHEP, 2000). This suggests that some bacterial pollution originates from contaminated stormwater runoff (NHEP, 2000). The greatest increase in bacterial concentration was seen in the Cocheco River where 87 fecal
coliiform colonies/100 ml sampled freshwater (79/100 ml tidal water) was seen during dry weather sampling and 550 fecal coliiform colonies/100 ml sampled in freshwater (272/100 ml tidal water) were seen following rainfall events. The high bacterial contamination seen even in the dry weather suggests ongoing sewage contamination, possibly due to cross-connections between sewer and stormwater systems (NHEP, 2000).

This research did not show a correlation between those areas classified as open or closed with the presence of *G. lamblia* and *C. parvum*. As seen in Table 4, fecal coliiform levels based on MPN analysis were 2 fecal coliiform colonies/100 ml sampled water, which is well below the acceptable limit. This observation supports finding in past studies that state that MPN analysis of fecal coliforms may not always indicate the presence or absence of other fecal pathogens (Burkhardt et al., 2000). The low MPN analysis seen in this work (2/100 ml) (Table 4) did not accurately evaluate the sanitary quality of the oysters.

**Site Results**

It is interesting to note that while *C. parvum* was detected at each sampling site (Table 4), *G. lamblia* was observed only at the Oyster River site during one sampling run. This may be attributed to the larger *Giardia* ovoid cysts settling out faster in the water column. These cysts settle into the sediment faster while the smaller *Cryptosporidium* oocysts are suspended in the water column for a greater period of time (Graczyk et al., 1999). Oysters are filter feeders, rather then detritus or sediment feeders, and would be able to collect and accumulate the suspended *Cryptosporidium* oocysts easier then the *Giardia* cysts, which have settled on the sediment. Additionally, the flow of water at
particular times of the year or sampling periods may impact the organisms’ rate of
disbursement through the water column. Depuration rates are not known for *G. lamblia*
and *C. parvum*. The possibility exists that there are different depuration kinetics for
oocysts and cysts. The oyster may have simply excreted the cysts from its system or
excreted enough cysts to fall below detection capability.

Figure 18 summarizes the multiplex assay for the environmental sample sites.
The presence of *G. lamblia* is indicated by the 211-bp amplicon and the presence of *C.
parvum* is indicated by the 600-bp amplicon. There was no cross binding seen between
primers of different organisms evidenced by lack of product formation in the negative
control lanes (Figure 18, lanes 2 and 3). To obtain a well-defined band for *C. parvum*, it
was necessary to increase the primer concentration from 1.0 μM to 1.5 μM. Numerous
hairpin loops and dimerization were predicted by the Primer Select program; however,
little interference by primer secondary structure was noted in the multiplex PCR assay.
Some excess bands were observed; however, there were no corresponding product
produced in the nested PCR.

Nested PCR produced results that corresponded to and confirmed those seen in
multiplex PCR (Figures 19 and 20). Figure 19, lane 3 shows the nested sample result for
*G. lamblia* (87-bp) with a negative control in lane 4. The nested PCR result for *C.
parvum* is shown in figure 20, lane 3, with the production of a predicted 97-bp band.
Lane one on both figure 19 and 20 shows the assay’s positive control, PCR product from
seeded samples.

Expected base pair sizes of 211-bp for *G. lamblia* and 600-bp for *C. parvum* in
the multiplex assay confirmed the presence of these pathogens in the site samples. These
results demonstrate that multiplex PCR can be a reliable means of detection and that any questionable bands produced can be confirmed or refuted using a subsequent nested PCR step.

The direct immunofluorescent technique was used during the second sampling collection as a confirmation of the presence or absence of *Giardia* cysts and/or *Cryptosporidium* oocysts. While this technique shows the presence or absence of these organisms, it is unable to distinguish pathogenic human strains from nonpathogenic strains of *Giardia* and *Cryptosporidium*. It is also problematic when analyzing environmental samples due to false positive results caused by cross-reaction with algae (Mayer, 1996). PCR results were confirmed with microscopic evaluation of the sample. Figure 24 shows *Cryptosporidium* oocysts (B) from the Nannie's Island site. This site also produced positive results via multiplex and nested PCR assays (Table 4). Figure 25 shows results from the Oyster River site. The presence of a *Giardia* cyst (A) and a *Cryptosporidium* oocyst (B) is evident. This confirms molecular results (Table 4).

A control sample of oysters was purchased from a local supermarket, original source of oysters unknown. The control sample did not show the presence of *G. lamblia* or *C. parvum* in either the multiplex PCR assay (Figure 21, lane 5) or the nested PCR assays (Figures 22 and 23, lane 3). A second sample, purchased at the same time, was seeded with $10^3$ cysts and $10^3$ oocysts to determine any inhibition. Multiplex bands were evident in the seeded sample (Figure 21, lane 4), showing that the oyster meat was not inhibiting molecular analysis.

The development of a multiplex PCR protocol is applicable to environmental sampling. The ethyl acetate sedimentation reduced the overall bulk of the sample,
allowing for the entire oyster to be analyzed rather than selected portions. The use of the immunomagnetic separation technique isolated and concentrated the *Giardia* cysts and *Cryptosporidium* oocysts from the oyster sample, removing environmental inhibitors as well as preventing inhibition from the oyster meat itself. The heat shock technique prevented the need for additional dissociation steps of the immunomagnetic beads, as required with immunofluorescent analysis of shellfish, and easily resulted in nucleic acid release. The multiplex PCR, utilizing primers specific for the human pathogens, provided rapid and sensitive amplification of *G. lamblia* and *C. parvum*. The steps required to optimize the multiplex reaction were reduced with the addition of the universal primer sequence to each primer. The increased sensitivity provided by the inclusion of nested PCR confirmed the multiplex results and reduced the potential for false positives. The protocol allowed for rapid and sensitive detection of protozoan pathogens in oysters harvested from the Great Bay.

There are several areas of potential contamination in the Great Bay Estuarine System, including human and wildlife impact (Figure 2, Table 1). While one may speculate what the possible source of contamination may be based on the potential sources of contamination, there are few solid data showing the concentration of *G. lamblia* cysts and *C. parvum* oocysts suspended in the water column. Other factors to consider include the flow of water, recent rain events, and any recreational water activity in the area that may impact the water column distribution.

Oysters in the Great Bay Estuarine System are harvested recreationally only. These harvested oysters may be consumed raw, thereby acting as an epidemiological agent in the spread of giardiasis and/or cryptosporidiosis. This work shows that *G.
*G. lamblia* and *C. parvum* can be detected in oysters harvested from the Great Bay Estuary using the developed multiplex procedure.

**Development of a Monitoring Program**

According to the New Hampshire Office of State Planning, the population density in Rockingham and Strafford counties is projected to show a 17% increase from 1998 to 2005 (NHEP, 2000). This will place added stress on the State’s resources. There may be added pollutants that will affect the quality of oysters in Great Bay due to the increasing pollutant loading from increasing development, particularly along the prime coastal property.

An increase in human population will also result in an increase in demand on shellfish resources. There is already a reduction in oyster density seen in the Great Bay. From 1991 to 1996, oyster density reductions in three beds of recreational importance ranged from 42% to 69% (NHEP, 2000). It is speculated that factors such as predation, limited substrates suitable for larval attachment, disease and a variety of management issues are factors in this decline (NHEP, 2000).

The results obtained from this research demonstrate that a monitoring program is feasible for the detection of *G. lamblia* and *C. parvum* in the eastern oyster using the molecular methods developed, multiplex PCR used in conjunction with nested PCR. These results could then be used to study depuration kinetics of *G. lamblia* and *C. parvum* in oysters and to gain further information to develop a risk assessment model to assess an acceptable level of risk to those who enjoy consuming raw oysters.
Depuration

Due to the problem of contamination, depuration may be one answer to averting a public health threat. It has been shown that *Escherichia coli* is broken down lysosmally in the digestive organs and used as a carbon source for the bivalve, while other bacteria are depurated within 48 hours of ingestion (Dore and Lees, 1995). Depuration studies on viruses show that they remain present in the digestive tract for much longer periods of time and are removed at a slower rate then bacteria (Burkhardt et al., 1992). So while there are data for depuration of virus from individual shellfish (Seraichekas et al., 1968), enterovirus accumulation and depuration by soft-shelled clams (Metcalf et al. 1979), bacterial and bacteriophage depuration (De Mesquita, 1991), there are no depuration data available as a guideline for shellfish contaminated with protozoans. There are no data that address whether the viability of these organisms affect the retention time or depuration rate.

A model that may be applicable to protozoans is one that investigated the accumulation of *Clostridium perfringens* (*C. perfringens*) by oysters (Burkhardt et al., 2000). *C. perfringens* is an anaerobic spore forming bacterial species and is resistant to disinfection and environmental stresses. This resistance is similar to what is observed with *G. lamblia* cysts and *C. parvum* oocysts. A recent study has shown that *C. perfringens* is able to bioaccumulate in oysters (Burkhardt et al., 2000). In fact, it is concentrated up to 245-fold higher in the oyster as compared with the overlying water (Burkhardt et al., 2000). This may suggest that the shellfish is unable to depurate the organism possibly due to the spore intertwining itself in the shellfish mucosa. There

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seems to be a demonstrated need for depuration data to provide reliable guidelines for safe and effective consumption conditions in post-harvest shellfish.

**Future Work**

This work has discussed and developed new means of detecting human pathogens in oysters. Development of a multiplex PCR assay that utilizes primer sequences having the 5' addition of the universal primer sequence, gives researchers the ability to include other pathogenic organisms into the protozoan multiplex. A reduction in optimization steps results in an easy and rapid means of detecting multiple organisms. Such organisms that could be incorporated in the multiplex include enteric viruses and pathogenic bacteria such as *Vibrio parahaemolyticus* and *Vibrio vulnificus*.

It has been noted that *Crassostrea virginica* (*C. virginica*) has antiviral and antibacterial agents in its tissue (Galstoff, 1964). These agents have been shown to inhibit *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhi* as well as various strains of influenza virus and poliovirus (Galstoff, 1964). This may be an interesting area of potential research as this may affect depuration conditions of various organisms. It is not known if *C. virginica* has antiprotozoal agents as well that may influence *G. lamblia* and *C. parvum* depuration or retention times.

There also needs to be more focus towards infectivity studies with *G. lamblia* and *C. parvum*. Multiplex techniques are essential for rapid and specific detection; however, viability is not established. Comparing the multiplex detection with infectivity studies would elucidate what happens to these organisms when ingested by shellfish. Some
factors include the ability of nonviable cysts and oocysts to elicit an immune response in some people and whether or not viability affects the rates of bioaccumulation in shellfish.

There also needs to be a re-evaluation of whether indicator organisms accurately predict the presence or absence of protozoa in shellfish. This is necessary for the development of an acceptable ongoing monitoring program. Organisms that are more resistant to stress, such as viruses or protozoa, may be more applicable as indicator organisms in the environment.

Research also needs to be done on the risk assessment of *G. lamblia* and *C. parvum*. The risk needs to be ascertained for the presence of these pathogens in environmental waters and oysters, rather than just potable water. For the purpose of developing risk models, however, more data and more sensitive techniques are needed since current data underestimate the number of cases (Gibson et al., 1998). The lack of documented outbreaks associated with *G. lamblia* and *C. parvum* in oysters may initially seem surprising. While cases of protozoan infection have been well documented in relation to drinking water (MacKenzie et al., 1994; Weniger et al., 1983), the study of *G. lamblia* and *C. parvum* in oysters is relatively new. There are many unknowns, including uncertainty if the high lipid content of oysters somehow masks the virulent effects of the protozoa. The nature of the illness also produces problems obtaining data for epidemiological investigations as the incubation period can range from one to 14 days, producing illness with varying disease severity from asymptomatic to severe (USFDA, 2000). As a result, many people may not seek medical attention. The lack of documented outbreaks in oysters may change as more research is done addressing climatic changes. The U.S. Global Research Program was developed in 1990 to
investigate concerns associated with climatic issues, particularly the intensity and frequency of rain events. Over the years 1997-2100, it is predicted that potential changes in salinity, estuarine water temperature, humidity and wind will impact the quality of water used for drinking, recreation as well as the quality of shellfish (Patz et al., 2000). Studies show a direct relationship between weather conditions and aquatic microorganisms including *Vibrio* species (Motes et al., 1983), viruses (Lipp et al., 1999), and red tide (Harvell et al., 1999). The concentration of *Giardia* cysts and *Cryptosporidium* oocysts in river water as well as incidence of disease outbreaks have also been directly attributed to rainfall events (Alterholt et al., 1998; Weniger et al., 1983). Along the East Coast an increase in marine related illness has been documented over the past 25 years, thought to be due to extensive precipitation resulting from El Nino (Harvell et al., 1999). This trend may continue resulting in documented shellfish-associated illness caused by *G. lamblia* and *C. parvum*, thus necessitating a need for monitoring techniques to be implemented.

As this research demonstrates, *G. lamblia* and *C. parvum* are present in estuarine environments, including the oysters harvested from those waters in approved areas in the Great Bay Estuarine System. Future work needs to focus on expanding the number of testing sites in the Estuary as well as the sampling times. An additional focus needs to be on exposure assessment to determine what an acceptable risk may be for people who enjoy eating raw oysters and what risk is unacceptable, possibly warranting shellfish bed closures.
LITERATURE CITED


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APPENDIX

Buffers and Solutions

1X PBS

9.785 g PBS powder (Sigma)

*Bring volume up to 1000 ml with reagent grade water. Autoclave 15 minutes. Store at 4°C. Final pH is 7.0.*

5X TBE

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boric Acid</td>
<td>27.5 g</td>
</tr>
<tr>
<td>Tris-base</td>
<td>54.0 g</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

*Bring volume up to 1000 ml with reagent grade water. Mix well. Store at room temperature.*