

**The Effects of E-Beam Irradiation, Microwave Energy and High Hydrostatic Pressure on
Presence and Health Significance of *Cryptosporidium parvum* in Eastern Oysters
(*Crassostrea virginica*)**

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(ABSTRACT)

Foodborne disease outbreaks associated with the protozoan parasite *Cryptosporidium* spp. are an emerging public health concern. Shellfish, including Eastern oysters (*Crassostrea virginica*) in Chesapeake Bay and other Atlantic coastal waters, have been identified as a potential source of *Cryptosporidium parvum* infection for humans. The inactivation of *C. parvum* and other pathogens in raw molluscan shellfish would provide increased food safety for normal and at-risk consumers. The objectives of this study were to evaluate the efficacy of three alternative food-processing treatments (e-beam irradiation, microwave energy, and high hydrostatic pressure processing) on the viability of *C. parvum* oocysts in Eastern oysters.

Oysters were artificially infected with the Beltsville strain of *C. parvum* and subjected to the three treatments in separate experiments. The effects of the treatments were evaluated by inoculation of the processed oyster tissues using the neonatal mouse bioassay.

E-beam radiation of in-shell and shucked oysters treated with doses of 1.0, 1.5 or 2.0 kGy produced significant reductions ($P < 0.05$) in *C. parvum* mouse infectivity. A dose of 2.0 kGy completely terminated the infectivity of *C. parvum* and did not adversely affect the visual appearance of the oysters.

Microwave treatments of shucked oysters at time (temperature) exposures of 1 sec (43.2°C), 2 sec (54.0°C), and 3 sec (62.5°C) produced a reduction in *C. parvum* mouse infectivity of 26.7%, 33.3%, and 46.7%, respectively. Microwave treatments at 2 sec (54.0°C) and 3 sec (62.5°C) showed extensive changes in oyster meat texture and color. Thus due to lack of efficacy and unacceptable tissue changes, microwave treatment of oysters is not considered a viable food processing method.

High pressure processing of shucked oysters at all pressures tested (305 MPa, 370 MPa, 400 MPa, 480 MPa, 550 MPa) significantly ($P < 0.05$) reduced infectivity of *C. parvum* oocysts

as measured by the neonatal mouse bioassay. A treatment of 550 MPa at 180 sec produced the maximum decrease of *C. parvum* infectivity (93.3%). The results indicate that HPP (high pressure processing) can produce significant ($P < 0.05$) reductions in infectivity of *C. parvum* oocysts. Measurement of tristimulus color values of pressurized raw oysters at extended processing times from 120 sec to 360 sec at 550 MPa showed an increase ($P < 0.05$) in whiteness.

One (e-beam irradiation) of the three food-processing treatments shows promise for commercial applications to reduce public health risks from cryptosporidiosis in Eastern oysters.

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CHAPTER I

INTRODUCTION

Foodborne disease outbreaks associated with the protozoan parasite *Cryptosporidium* spp. are an emerging public health concern. Since 1993, several foodborne outbreaks of cryptosporidiosis have shown the threat from *Cryptosporidium* is not limited to waterborne outbreaks (Millard et al., 1994; Rose and Slifko, 1999; Slifko et al., 2000). Concerns about *Cryptosporidium* first arose due to waterborne outbreaks of cryptosporidiosis, including a 1993 outbreak that sickened more than 400,000 people and killed over 100 people in Milwaukee, Wisconsin (MacKenzie et al., 1994; Kramer et al., 1996; Rose et al., 1997). Cryptosporidiosis is a cause of gastrointestinal illness in humans and domestic animals worldwide (Olson et al., 1999) and is a significant contributor to mortality of immunocompromised or immunosuppressed persons, such as AIDS patients (Fayer, 1997). Cryptosporidiosis has caused massive waterborne epidemics around the world (Rose et al., 1997).

Cryptosporidium is widely distributed in the surface waters in the U.S. and Canada (Rose et al., 1997; Lechevallier et al., 1991; Levine and Craun, 1990). Significant sources of *Cryptosporidium* include wastewater treatment facilities and animal farms, especially ruminant farms (Fayer et al., 1997b; Graczyk, 1997; Rose et al., 1997). Rainfall, floods, and sewage overflows wash *Cryptosporidium* oocysts into rivers, bays, and oceans and contaminate fresh and marine waters with *Cryptosporidium* oocysts. The Chesapeake Bay is a prominent example of susceptibility to environmental contamination with *Cryptosporidium parvum* (Fayer et al., 1998; Graczyk et al., 2000). *Cryptosporidium*-contaminated water can be introduced into the food supply in various ways, such as water used in food preparation or processing (e.g. fruit juices), or as water ingested or filtered by organisms (e.g. shellfish).

Researchers detected *Cryptosporidium parvum* oocysts in Eastern oysters (*Crassostrea virginica*) all commercial oyster harvesting sites tested in Maryland tributaries to Chesapeake Bay (Fayer et al., 1998; Graczyk et al., 2000). Because oysters are filter feeders and are often consumed raw, the researchers concluded that *C. parvum* oocysts in oysters pose a potential public health problem as a possible source for the foodborne-disease. Additional research found

Cryptosporidium species in commercial shellfish (oysters and/or clams) from 64.9% of sites sampled in 13 Atlantic coast states from Maine to Florida and New Brunswick, Canada (Fayer et al., 2003).

The development of food processing treatments to reduce or eliminate *C. parvum* oocysts in oysters could help protect shellfish consumers from this pathogen. Because thermal processing can reduce the sensory qualities that make raw oysters so valuable, the challenge is to discover if non-thermal processing techniques can reduce or eliminate *C. parvum* oocysts in raw oysters while still retaining their sensory qualities. For example, high hydrostatic pressure is especially suited for use with fish and shellfish because it provides a non-thermal treatment of food pathogens with an extended shelf life while preserving the sensory qualities of the seafood (Flick, 2003).

The objectives of this study are to test the efficacy of three alternative food processing treatments (e-beam irradiation, microwave energy, and high hydrostatic pressure processing) on the viability of *C. parvum* oocysts in Eastern oysters using the neonatal mouse bioassay (Lindsay et al., 1999).

LITERATURE REVIEW

2.1 *Cryptosporidium parvum*

Cryptosporidium parvum is an apicomplexan parasite with worldwide distribution that causes acute diarrhea in immunocompetent individuals and life-threatening illness in immunocompromised or immunosuppressed individuals (Current and Garcia, 1991). *Cryptosporidium* causes gastrointestinal illness in more than 152 mammals and other vertebrates (Fayer et al., 2000). The first reported case of bovine cryptosporidiosis was in 1971 (Fayer, 1997). Interest increased in the 1980s when the Centers for Disease Control and Prevention reported that 21 patients with Acquired Immune Deficiency Syndrome (AIDS) had become ill with cryptosporidiosis (Centers for Disease Control and Prevention, 1982). Waterborne and foodborne outbreaks of cryptosporidiosis have raised increasing public health concerns. It has emerged as a global human health problem facilitated in its spread by waterborne and foodborne transmissions of the parasite. The parasite causes cryptosporidiosis, a chronic and life-threatening illness for immunocompromised and immunosuppressed individuals due to lack of effective prophylaxis or therapy (Fayer et al., 1997b). *Cryptosporidium* is recognized today as one of the most common opportunistic parasites (Guerrant, 1997). Therefore, high-risk groups are advised to avoid exposure to *C. parvum*.

Historically, two genotypes of *C. parvum* have been identified by molecular techniques (Sulaiman et al., 1998). The “animal adapted” or “zoonotic” genotype (genotype 2, *C. parvum* bovine genotype) has been shown to be transmissible among cattle, pigs, sheep, mice, and humans and may infect other mammalian species including domestic and wildlife species. The “human adapted” genotype (genotype 1, recently named *C. hominis*) is thought to cycle within the human population (Sulaiman et al., 1998; Morgan et al., 1997; Xiao et al., 1999) but is not infectious to other vertebrate groups (Casemore et al., 1997). Based on biological and molecular data, *Cryptosporidium* in human intestines was proposed to be a new species *Cryptosporidium hominis* (Morgan-Ryan et al., 2002). Both *Cryptosporidium* species can produce life-threatening infections in people with impaired immune systems (Current and Garcia, 1991).

Cryptosporidium parvum oocysts are able to endure environmental stress, and so create a high probability of reaching a new susceptible host. Major sources of environmental contamination by the oocysts are wastewater treatment plants, discharge of untreated sewage, infected persons,

companion animals, and run-off from farm animals, particularly cattle farms (Fayer et al., 1997a; Graczyk, 1997; Graczyk et al., 1997; O'Donoghue, 1995; Rose et al., 1997).

The oocysts from the contaminant source are transported by surface run-off to areas where exposure to potential new hosts occurs, such as bathing areas, extraction points for drinking water, and watering areas for livestock or wildlife. Dispersal of oocyst contamination is governed by hydrodynamic, chemical, and biological factors, i.e., water flow, attachment of freely suspended oocysts to particles, sedimentation and resuspension of free and attached oocysts, and survival of oocysts (Medema et al., 1998b).

Symptomatic *C. parvum* infection is most prevalent in the young, such as children and bovine calves, and the immunocompromised, such as AIDS patients (Crawford and Vermund, 1988). A relatively low infective dose (ID) of *C. parvum* (ID₅₀ of 134 oocysts) is considered to be the infective dose for a healthy population, and approximately 30 oocysts may initiate infection (DuPont et al., 1995). An infected calf can excrete over a billion oocysts daily during peak oocysts shedding (Harp et al., 1990). Guerrent, 1997 reported that *Cryptosporidium* infection in 2.1% to 6.1% of immunocompetent persons in industrialized and developing countries. Some distinct differences in oocyst shedding patterns occurred between *C. parvum* human genotype, (*C. hominis*) and *C. parvum* (bovine) (McLauchlin et al., 1999; Xiao et al., 2001).

Rose et al. (1997) examined surface water in the United States and Canada and reported the prevalence of oocysts at 100% with concentrations as high as 5,800 oocysts per liter. Water transmission of this parasite has led to large outbreaks of cryptosporidiosis, with increasing frequency since the 1980s. One of the largest outbreaks occurred in Milwaukee, Wisconsin in 1993 and involved the infection of 400,000 individuals (MacKenzie et al., 1994; Centers for Disease Control and Prevention, 1996). Eventually, over 100 people died in the Milwaukee outbreak of cryptosporidiosis (Rose, 1997). However, many less serious outbreaks have been reported in the United States and Canada (Moore et al., 1993). Outbreaks related to *Cryptosporidium* in drinking water primarily were linked to the spreading of viable *C. parvum* oocysts through drinking water purification system process (Fayer et al., 2000). In the United States, in 1984 an outbreak affected 2006 persons in Braun Station, Texas (D'Antonio et al., 1995); 12,960 residents had gastrointestinal illness during water outbreak at Carroll County, Georgia, in 1987 year (Hayes et al., 1989); in 1992, 15,000 residents of Jackson County, Oregon

became ill with gastroenteritis during an outbreak related to the treatment deficiencies at the regional water plant facilities (Leland et al., 1993; Moore et al., 1993). Unfiltered spring and ground water conditions in Ogose, Japan led to an outbreak in 1996 that affected more than 9000 people (Anon., 1996a). *Cryptosporidium* has become recognized as the most important biological water contaminant in the United States (Rose et al., 1997). Cryptosporidiosis outbreaks associated with the consumption of food were first reported from freshly pressed (unpasteurized) apple cider (Millard et al., 1994), unpasteurized apple juice (Anon., 1996b), and chicken salad (Centers for Disease Control and Prevention, 1986).

Cryptosporidium parvum oocysts are small (4-6.0 μm) and their infectivity is long-lasting in aquatic environments (Fayer et al., 1997a; Fayer et al., 1998; Robertson et al., 1992; Rose et al., 1997). The oocysts are continuously (as distinct from intermittently) prevalent in surface waters (Hansen and Ongerth, 1991; Rose et al., 1997).

2.1.1 *Cryptosporidium* in oysters

Some research findings (Fayer et al., 1997a) demonstrated that oysters in natural waters harbored infectious *C. parvum* oocysts and could serve as a mechanical vector of the pathogen. Tamburrini and Pozio (1999) reported that *C. parvum* could survive in seawater for at least 1 year and can be filtrated out by benthic mussels, retaining their infectivity up to 14 days. Graczyk et al. (1998a) exposed Asian freshwater clams (*Corbicula fluminea*) for 24 hours to water contaminated with infectious *C. parvum* oocysts and that extracted oocysts in clam tissues and feces on days 1 through 14 were infectious for neonatal BABL/c mice on day 7. They concluded that both seawater and shellfish could be a source of infection.

Researchers detected *C. parvum* oocysts in Eastern oysters (*Crassostrea virginica*) at all commercial oyster harvesting sites tested in Maryland tributaries to Chesapeake Bay (Fayer et al., 1998; Graczyk et al., 2000). Because oysters are filter feeders and are often consumed raw, the researchers concluded that *C. parvum* oocysts in oysters pose a potential public health problem and possible source for foodborne-disease cases of cryptosporidiosis. A more recent and geographically wider survey found *Cryptosporidium* species in commercial shellfish (oysters and/or clams) from 64.9% of sites sampled in 13 Atlantic coast states from Maine to Florida and New Brunswick, Canada (Fayer et al., 2003). Reports of food related outbreaks of this emerging disease are difficult to document and probably under-reported (Fayer et al., 2000).

Because of these findings, the safety of raw shellfish consumption has been a concern of the U.S. Food and Drug Administration, various consumer protection organizations, consumers, seafood trade organizations, and the food service industry. In accordance with rising public health concern, cryptosporidiosis was evaluated for active surveillance in 1997 under the Foodborne Disease Active Surveillance Network program. The parasite is able to survive in the tissue of fresh shellfish from harvest through consumption and is resistant to treatment with chemicals (liquids and gasses) (Campbell et al., 1993; Ernest et al., 1986; Chauret et al., 2001; Fayer et al., 1996; Ransome et al., 1993) and refrigeration temperatures (Fayer and Nerad, 1996).

While the presence of *C. parvum* and its potential public health implications in raw shellfish are important, the food safety issues associated with processed and prepared products are equally important for several reasons. First, most shellfish are consumed processed and prepared rather than raw. Second, at-risk individuals need to know the health risk associated with the food products they consume. For both raw and processed shellfish it is important to identify how alternative processing operations (e.g., e-beam irradiation, microwave energy, and high hydrostatic pressure) might reduce survival of *C. parvum* oocysts in Eastern oysters. For raw oysters it is important to find the degree to which alternative process might control *C. parvum* oocysts in Eastern oysters and still allow the oysters to be considered a fresh seafood product.

2.1.2 Methods of detecting *Cryptosporidium*

Analytical methods reported for isolation and detection of *C. parvum* oocysts include immunofluorescence assay (IFA) methods (Rochelle et al., 1997; Stinear et al., 1996; Graczyk et al., 1998b); flow cytometry (Medema et al., 1998a; Rochelle et al., 1997; Kaucner and Stinear, 1998); laser scanning and IMS (immunomagnetic separation) (Reynolds et al., 1999); polymerase chain reaction (PCR) analysis (Deng and Cliver, 1998); continuous flow centrifuge (Swales and Wright, 2000), and HCT-8 cell culture (human ileocecal adenocarcinoma cells) infectivity assay (Chappell, 2000). These methods were applied for detection of *C. parvum* from fruits and vegetables (Robertson and Gjerde, 2001) where 42% recovery of *C. parvum* was reported from salad crops. Detection of *C. parvum* in dairy products such as yogurt, low fat milk and ice-cream (Deng and Cliver, 1999) approached to 82% recovery. Applications of homogenization for separating oocysts from the food matrix limit the application in solid food products (vegetables, meat, shellfish) because of interference from food debris with oocysts leading to an overall reduction in total oocyst recovery (Moriarty et al., 2004).

Investigators have reported acceptable results for detecting of *C. parvum* oocysts using the IFA method combined with the polymerase chain reaction (PCR). These two techniques have been used in the analysis of Eastern oysters (Graczyk et al., 1998b; Fayer et al., 1998) and freshwater clams (Graczyk et al., 1998b). The IFA method yields interval data (number of oocysts present) while the PCR method includes greater sensitivity, rapid analysis of many samples, relatively low cost, simultaneous detection of pathogens, and ability to discriminate between species and strains (if suitable primers are selected).

An HCT-8 cell culture (human ileocecal adenocarcinoma cells) infectivity assay provides evaluation of oocysts excystation and sporozoite viability (Phelps et al., 2001). This assay tests for *C. parvum* viability (Chappell, 2000) because it identifies the development stages of parasite on cell model and mimics human infection.

Researchers have assessed oocysts viability with the use of fluorogenic vital dyes (*in vitro*), 4',6-diamidino-2-phenyl indol (DAPI) and propidium iodine (PI) (Campbell et al., 1992), and incorporation of PI staining (Dowd and Pillai, 1997) with an indirect fluorescent antibody detection method. Combination of these two detection methods (DAPI and PI staining) provides simultaneous measurement of oocysts presence and viability. Campbell et al. (1992) showed close correlation between this detection method (*in vitro*) and infectivity in a mouse assay (*in vivo*) for *Giardia muris* oocysts. For *C. parvum* viability and infectivity, the neonatal mouse model remains the first choice among *in vivo* tests (Lindsay et al., 1999; Bukhari et al., 2000).

2.2 E-beam radiation

Ionizing radiation is used to eliminate the biological hazards through the reduction or elimination of food poisoning microorganisms and parasites. Ionizing radiation includes gamma rays, x-rays and e-beam radiation. Gamma rays, x-rays and e-beam radiation produce electrons that form positively and negatively charged ions (ionization) in the irradiated sample resulting in chemical and biological effects in biological systems.

The radiation dose absorbed by the food product is measured in units of Gray (Gy), which is equal to the absorption of 1 joule of energy per kilogram of food. One Gray equals 100 rads; 10 kGray equals 1 Megarad (rad is an older unit of measurement). A D-dose is the amount of irradiation that it takes to destroy 90% of the organisms or one decimal log.

Because of the potential benefits to public health and safety, food irradiation has been endorsed by many national and international organizations, such as the World Health

Organization, the American Medical Association, the Council on Agricultural Science and Technology, and the Institute of Food Technologists (Molins, 2001a).

Gamma rays and x-rays can penetrate deeply into food products. In contrast, electrons generated by e-beam-machines have some mass, and thus, can penetrate less deeply than gamma rays and x-rays. However, because e-beam radiation does not use radioactive sources, it has practical advantages in terms of processing equipment, safe operation, and potentially, consumer acceptance of irradiated foods (Murano, 1995).

Another difference between gamma rays and e-beam radiation is the dose rate. According to Hayashi (1991), the dose rates for gamma rays from commercial Cobalt-60 sources are 1-100 Gy/min, while the dose rates for electron beam accelerators are 10^3 - 10^6 Gy/sec. While the dose rate is usually not considered critical in food irradiation, there are reports that dose rate can influence the effect of irradiation on food and living organisms (Hayashi, 1991). As a result, the differences in effects of food irradiation between gamma rays and e-beam have been attributed to the difference in dose rate rather the type of radiation (Hayashi, 1991).

A study comparing e-beam versus gamma rays as alternative sources for irradiation processing found that both types of radiation produced similar chemical and biological effects, but that differences in physical characteristics and economics may favor one over the other for a particular food process (Cleland and Pageau, 1985). However, when both e-beam and gamma rays produce the necessary dose distribution to treat the product, e-beam accelerators have advantages over gamma rays sources in terms of total costs (capital, operating and maintenance costs), especially for smaller facilities (Cleland and Pageau, 1985).

2.2.1 Irradiation and shellfish

Mallet et al. (1991) conducted irradiation survival studies on bivalve mollusks *C. virginica*, *Mya arenaria*, and *Mercenaria mercenaria*, subjected to a dose range of 0.2 to 7 kGy from a 0.8 megaCurie Cobalt source. A sample set of 50 oysters (*C. virginica*) showed no significant differences in six-day survival times at doses up to 2.5 kGy. Samples exposed to 3.0, 5.0 and 7.0 kGy showed radiation effects with median failure after 11 days of exposure. These results suggested an acute lethal dose as 3.0 kGy.

In regard to sensory effects based on taste tests, Mallet et al. (1991) found the oysters were not susceptible to radiation induced sensory changes. The sensory quality of oysters was of acceptable quality at doses up to 3.0 kGy. Mallet et al. (1991) also tested the viral inactivation

produced by irradiation of *C. virginica*. Live oysters were injected with poliovirus 1, simian rotavirus SA-11, and hepatitis A virus to determine gamma doses necessary to achieve a log cycle reduction (D_{10}) of the viruses. Based upon the irradiation treatments, the inactivation dose (D_{10}) calculations for the oysters averaged 3.1 kGy for poliovirus 1, 2.4 kGy for simian rotavirus SA-11, and 2.02 kGy for hepatitis A virus.

In discussing the research results, Mallet et al. (1991) noted that the capacities of oysters and other intertidal bivalve mollusks to sustain intense radiation exposure are exceptional. These authors speculated these bivalves are predisposed to radiation resistance due to their unique anatomical and physiological adaptations to intertidal life, such as:

1. Facultative anaerobic metabolic capacities;
2. Minimalization of body lipid stores;
3. Anatomically simplified nervous system;
4. Metabolic production of radioprotective agents; and
5. Low metabolic rate and low cell division rate.

Mallet et al. (1991) concluded that irradiation, when used as a supplement to conventional depuration, has a high potential to significantly enhance shellfish sanitation. However, they cautioned against using irradiation in an attempt to market shellfish of inferior microbial quality. Irradiation should be used to increase consumer safety of shellfish that meet sanitary standards, such as those of the National Shellfish Sanitation Program.

Irradiation has been shown to extend shelflife of fresh shellfish and reduce bacteria. Kilgen (2001) reported on the effects of low, medium and high doses of ionizing radiation on fresh, frozen, and processed seafood products. It was noted that, 1) in general, Gram-negative bacteria are more sensitive to ionizing radiation than Gram-positive bacteria, and 2) a dose of 4 kGy was found sufficient to eliminate non-spore-forming pathogens in many kinds of foods, including frozen seafood. In regard to *C. virginica* oysters, Kilgen (2001) reported on the radiation dose necessary to reduce bacterial numbers to a non-detectable level. These studies found that level of 1.5 kGy to eliminate *Escherichia coli*; 1.2 kGy to eliminate *Vibrio* spp.; 1.5 kGy to eliminate *V. vulnificus*; 1.0 kGy to eliminate *V. cholerae*; 1.0 kGy to eliminate *V. parahaemolyticus* seeded in *C. virginica*. Alternative food processing treatments may be implemented in seafood processing if they provide a 5-log reduction of the pertinent pathogen. Tauxe (2001) reports that the elimination of 99.999% of bacteria (a so-called 5-logarithm kill)

takes 5 times the irradiation dose needed for a 1 log kill and would reduce a million bacteria to ten. For example, to reduce *Campylobacter* in meat it takes 0.2 kGy with one decimal log or 1 kGy to reduce it by 5 decimal logs.

Jenkins et al. (1995) showed that gamma irradiated and nonirradiated *Eimeria tenella* sporozoites exhibited differential uracil uptake and expression of 7- to 10-kDa metabolic antigen. Metabolic 7- to 10-kDa antigen expression by 25 kRad (2.5 kGay) irradiated sporozoites was greatly reduced compared to nonirradiated or 15 kRad (1.5 kGay) irradiated intracellular sporozoites. The findings suggested that the metabolic 7- to 10-kDa antigen plays a role in protective immunity elicited by nonirradiated or 15 kRad (1.5 kGay) irradiated *E. tenella* sporozoites.

Jakabi et al. (2003) examined the effects of ionizing radiation on inactivation of *Salmonella enteritidis*, *Salmonella infantis*, and *V. parahaemolyticus* in oysters (*C. brasiliensis*) using gamma radiation (^{60}Co) in doses ranging from 0.5 to 3.0 kGy. They found that a dose of 3.0 kGy was generally sufficient to reduce the level of *Salmonella* serotypes by 5 to 6 log₁₀ units. A dose of 1.0 kGy was sufficient to produce a 6-log₁₀ reduction in the level of *V. parahaemolyticus*. The highest irradiation dose (3.0 kGy) did not kill the oysters or affect their sensory attributes. They considered a dose of 3.0 kGy as effective in inactivating *Salmonella* and *V. parahaemolyticus* without changing oyster odor, flavor, or appearance.

2.2.2 Effects of e-beam radiation on parasites

The potential use of irradiation to control infectivity of food-borne parasites has been the subject of increasing research over the past 20 years. Most research has used gamma radiation. Previous research has shown that the high-energy ionization, whether produced by gamma, x-ray or e-beam radiation, has generally similar chemical and biological effects (Wilkinson and Gould, 1998). Therefore, the results of gamma radiation research to control infectivity of food-borne parasites are relevant to the potential use of e-beam radiation to control infectivity of food-borne parasites.

Based on a review of radiation research, Molins (2001b) concluded that parasites of public health significance are far more sensitive to radiation than either bacteria or viruses. Previous research has demonstrated that ionizing radiation is highly effective at eliminating parasites from meat and poultry (Molins, 2001c). The irradiation process achieved these results with only minimal increase in temperature and without significant change in physical, chemical,

and sensory properties. These results make ionizing radiation ideal for decontamination of muscle foods (IAEA, 1996). Wilkinson and Gould (1998) and the World Health Organization (1999) reviewed the results of research on the effect of irradiation on parasites. The effects of irradiation on fish and crustacea are summarized in Table 1.

Table 1.

The effect of irradiation on parasites in fish and crustacea ^a

Parasite	Occurrence/ mode of infection	Dose (kGy)	Effect of irradiation
<i>Angiostrongylus cantonensis</i>	Parasitic nematode found in mollusks, shellfish	2	Minimum effective dose
<i>Anisakis</i> spp.	Nematode ingested if fish is eaten raw or lightly salted	2-10	Reduce infectivity of larvae
<i>Chlonochris</i> spp.	Chinese liver fluke, occurs in raw fish	0.15	<i>In vitro</i> minimum effective dose
<i>Gnathostoma spinigerum</i>	Parasitic nematode found in fish	7	Reduces nematode recovery rate in mice
<i>Opistorchis viverrini</i>	Liver fluke found in contaminated raw, pickled or smoked fish	0.1	<i>In vitro</i> minimum effective dose
<i>Paragonimus</i> spp.	Parasitic trematode found in crabs and crayfish in Asia	0.1	<i>In vitro</i> minimum effective dose

^a Adapted from World Health Organization (1999) and Wilkinson and Gould (1998), based on IAEA (1993) and Roberts and Murrell (1993).

Other related research found that a dose of 0.25 kGy killed all viable cysts of the protozoan *Entamoeba histolytica* (Schneider, 1960), and a dose of 0.37 kGy prevented development of the egg-producing stage of the tapeworm *Hymenolepis nana*.

The research (Table 1) showed that irradiation has been successful in controlling infectivity of various parasites in the fish, crustacea and meat. The minimum dose to control infectivity varied from 0.1 to 10 kGy depending on the specific parasite and host tested.

Yu and Park (2003) investigated the effect of gamma irradiation on the viability of *Cryptosporidium parvum* oocysts. A combined indirect immunofluorescence and nucleic acid staining and animal infectivity study was used to determine oocyst viability. The study suggests that at least 50,000 Gy (50 kGy) of gamma irradiation is necessary for the complete elimination of oocyst infectivity in mice (Yu and Park, 2003).

2.3 Microwave energy

The use of microwave irradiation for sterilization and pasteurization of food products is a common practice for the commercial food industry (Schlegel, 1992). Microwaves are electromagnetic waves that have both an electric field and a magnetic field. The electric field is the main source of heating, produced by rotation of polar molecules that generates heat by molecular friction. Microwaves are the part of the electromagnetic spectrum with radiation in the frequency range of 300 million cycles per second (300 MHz) to 300 billion cycles per second (300 GHz), with wavelengths varying from 1 m down to 1 mm.

In the United States, commercial microwave ovens generally operate at a frequency of 2450 MHz; in some other countries, a frequency of 915 MHz is used (Heddleson and Doores, 1994). The penetration depth of microwaves into food increases as the wavelength increases (as the frequency decreases) (Annis, 1980).

In contrast to ionizing radiation by electron beams, microwaves produce nonionizing radiation and are too weak to break chemical bonds. The microwave radiation is absorbed at the molecular level, and results in heating and vibration changes of the molecules. Also in contrast to e-beam treatment of food, microwave energy treatment of food is familiar to consumers. Microwave ovens are common food appliances in homes and offices.

Many studies have shown that microwave heating of food is effective in destroying bacteria and pathogenic microorganisms. Fujikawa and Ohta (1994) reported on the patterns of destruction of several kinds of bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas fluorescens*) by microwave irradiation. Heddleson et al. (1993) reported on the destruction of *Salmonella* species heated in aqueous salt solutions by microwave energy.

Microwave treatment of food produces thermal and non-thermal effects in the food. The thermal effects from microwave heating have some differences from conventional heating. For example, Khalil and Villota (1988) studied the effects of microwave heating and conventional heating on *Staphylococcus aureus*. They found sublethal microwave treatment (30 minutes at 50°C) resulted in greater cell injury than conventional heating. They also found conventional heating produced significantly more enterotoxin A (1-2 ug/ml) in recovering cells than microwave treatments for all time periods (0-72 h). They concluded that microwave treatment produced greater damage to cells and membranes than conventional heating, and they attributed the major part of the injury to thermal effects.

Compared with conventional heating, for example using hot water, microwave heating has the advantage of providing heat transfer without a heating medium. In addition, microwave heating takes less time to reach critical temperatures and has a rapid rate of volumetric heating. One disadvantage of microwave treatment can be uneven heating, resulting in hot and cold spots. For example, surface heating effects in which the food surface reaches a higher temperature than the center of the food are more of a problem with microwave ovens operating at 2450 MHz than at 915 MHz (Mudgett, 1989; Heddleson and Doores, 1994). For destruction of microorganisms in food, it is especially important to discover the locations of hot and cold spots for a particular food. Destruction is exponentially related to temperature since small differences in temperature may make large differences in the number of microorganisms destroyed.

In a review of research on microwave-induced destruction of foodborne pathogens, Heddleson and Doores (1994) pointed many factors that influence microwave thermal processes (mass of objects, shape of objects, specific heat, thermal conductivity, etc.) are the same as conventional heating. But other factors, such as moisture content and salt content have a greater role in microwave heating than in conventional heating. Microwave heating has been used to destroy microorganisms in food. However, there is a long-standing debate about the thermal and non-thermal effects produced by microwave treatment of food (Anantheswaran and Ramaswamy, 2001). A recent review of the controversy over whether microwave produces biological effects by non-thermal effects as well as thermal effects was published by Banik et al. (2003). The investigators concluded that these recent studies do indeed show that microwave can athermally induce various physiological effects. However, the research results do not always specify the mechanism to account for the non-thermal effects.

As previously discussed, several studies have been conducted on the use of microwaves to control bacteria and some pathogens, but there are significant gaps in research. For example, Doyle and Mazzotta (2000) reported that heat resistance data for *Salmonella* are still nonexistent or scarce in chicken meat, fruit juices, and aquacultured fish.

2.3.1 Effects of microwave energy on parasites

Conder and Williams (1983) studied the effects of microwaves on developmental and infective stages of three parasites: *Eimeria nieschulzi* (protozoa), *Strongyloides ratti* (nematode) and *Taenia taeniaeformis* (cestode). Microwave radiation was applied to laboratory preparations and fecal samples containing the three parasites. The results showed that microwave radiation

was extremely effective in killing or preventing development of helminth and protozoan parasites without unduly distorting eggs or developmental stages. While this early study was aimed at laboratory applications (decontaminating parasitological specimens and sterilizing laboratory glassware), it suggests that research into the effects of microwave radiation on decontaminating food may be promising.

Bouchet and Boulard (1991) studied the effect of microwaves on the ultrastructure of oocysts of *Eimeria magna*. The oocysts were exposed to microwave energy (2,450 MHz; 600 W) for different durations (10, 15 and 20 sec). The microwave treatments progressively destroyed the three layers of the oocyst wall; the innermost layer was destroyed first. Microwave treatments also damaged internal structures, producing swollen mitochondria, a loss of ribosomes, and fragmentation of the rough endoplasmic reticulum. The wall-forming bodies were no longer identifiable. Bouchet and Boulard (1991) recommended more research into the biological effects of microwaves in order to improve our understanding of the consequences of such treatment and to investigate its utility in the control of transmissible pathogenic organisms.

Adams et al. (1999) studied the relationship between temperature and survival of nematodes of the species *Anisakis simplex* in microwave-processed arrow-tooth flounder (*Atheresthes stomias*). Fillets, 14 cm long, 4.5 cm wide, and approximately 1.75 cm high, were inoculated with 10 live nematodes and then microwaved. No viable nematodes survived microwave processing at 160° F (71°C) or 170 °F (77°C). Smaller fillets required only 150° F (65°C) probably due to fewer cold spots during microwave processing. In contrast, larger whole fillets required 170° F (77°C). Inactivation of the parasites was attributed to the thermal effects of the microwave treatment (Adams et al., 1999).

2.4 High pressure processing

The pioneering work of Hite (1899; 1914) demonstrated the potential of high pressure processing (HPP) to destroy microorganisms and preserve milk, fruits, and vegetables. Major commercial use of HPP for food processing began in 1990 in Japan on products such as jams, fruit juices, fruit toppings, and tenderized meat. Since 1990 food researchers and the food industry have found increasing uses for HPP for a variety of food. This emerging technology has been applied to both raw and processed fish and shellfish (Flick, 2003).

High pressure processing, also referred to as high hydrostatic pressure (HHP) or ultra high pressure (UHP) processing, applies pressures in the range of 100 to 800 MPa to liquid or

solid foods, with or without packaging (Farkas and Hoover, 2000). Food items, often in final consumer packaging, are placed in a pressure vessel, water is added, the vessel is closed, and the contents are pressurized. Water is the most common medium that transmits the pressure. Because the water pressure is equal in all directions (hydrostatic), food items with high water content are not crushed (Anstine, 2003).

The exposure time for application of HPP in commercial food processes can vary from a millisecond to minutes, with 20 minutes as the practical upper limit due to economic considerations (Farkas and Hoover, 2000). High pressure processing is often used as a batch process for pre-packaged foods, but HPP also can be used in a semi-continuous process for pumpable liquids (Barbosa-Canovas and Rodriguez, 2002).

The compression of water in the HPP pressure vessel will increase food temperature about 3 degrees C per 100 MPa (Farkas and Hoover, 2000). This adiabatic heating (an increase in temperature due to an increase in pressure) varies depending on food composition, with a larger increase for fatty foods. When the vessel decompresses, the food product cools to the original temperature, provided no heat has been gained or lost from the walls of the vessel.

The adiabatic heating associated with HPP is very small compared to heating associated with thermal processing of food. As a result, HPP leaves the sensory and nutritional quality of food virtually unaffected compared with thermal processing. High pressure processing is considered non-thermal processing and is increasingly used for food processing because it provides food products with better quality than traditional thermal processes (San Martin et al., 2002). Other advantages of HPP include reduced processing times, no vitamin C loss, retention of flavor, texture, and color (Tewari et al., 1999).

Another effect of the HPP is the compression of food in the HPP pressure vessel, resulting in a temporary decrease in the volume of the food. When the vessel decompresses, the food product returns to its original volume. If the food is in a package, the package must be able to accommodate up to a 15% reduction in volume and subsequent return to original volume without breaking the package seal and barrier properties (Farkas and Hoover, 2000).

High pressure processing produces a variety of biological effects on microorganisms and inhibits foodborne pathogens in certain foods (Hoover et al., 1989). This process can cause changes in cellular morphology. Pressure can collapse intracellular gas vacuoles. High pressure can cause structural changes that result in cessation of movement by motile organisms, especially

protozoa. Pressures in the range 300 to 450 atm (30.4 to 45.6 MPa) applied to *Pseudomonas* produced a variety of morphological changes including separation of the cell wall from the cytoplasmic membrane and a decrease in the number of ribosomes. Pressure and its effect on volume result in damage to many cellular processes, and most likely, inhibit or destroy a combination of cell processes or functions.

The application of pressure results in changes of the pH of seawater. The pH of seawater at 1 atm and 1 degree C is 8.10, but at 1,000 atm (101.3 MPa) the pH decreases to 7.87 (Dring, 1976). The application of pressure changes the pH of the medium and reduces the pH range for microbial growth (Marquis, 1973). For example, Marquis (1973) found that growth of *Streptococcus faecalis* is inhibited by a pH of 9.5 at atmospheric pressure and by a pH of 8.4 at 400 atm (40.5 MPa). Research indicates that pH is a significant factor in influencing the sensitivity of an organism to pressure (Hoover et al., 1989).

High pressure processing can cause changes in biochemical reactions. The application of pressure retards reactions involving volume increases, and enhances reactions involving volume decreases (Hoover et al., 1989). The application of HPP on raw oysters produced some flattening of form but enhanced the flavor, possibly due to the infusion of the salty liquor in which the oysters were pressurized (Hoover et al., 1989).

High pressure processing inactivated *V. parahaemolyticus* and *V. vulnificus* in pure cultures and whole Eastern oysters (*C. virginica*) (Koo et al., 2002; Cook, 2003). High pressure processing reduced microbial activity in Pacific oysters (Calik et al., 2002) and also shucked oysters (He et al., 2002).

2.4.1 Effects of high pressure processing on parasites

The development of food processing treatments to reduce or eliminate *C. parvum* oocysts in oysters could help protect shellfish consumers from this pathogen. High pressure processing is a treatment that has been reported to control infectivity of foodborne pathogens in various foods (Tewari et al., 1999). High pressure processing is especially suited for use with fish and shellfish because it provides non-thermal treatment of food pathogens and longer shelflife while preserving the sensory qualities of the seafood (Flick, 2003).

Anisakis simplex, a common nematode in many marine fish, is the causative agent for fishborn parasitoses. Molina-Garcia and Sanz (2002) showed that a lower pressure of 200 MPa

for 10 min and 140 MPa for up to 1 h could be successfully employed to kill all *Anisakis* larvae suspended in either distilled water or a physiological isotonic solution.

The HPP treatments of finfish, such as raw salmon and arrowtooth flounder (207 MPa/180 sec, 276 MPa/90-180 sec, 414 MPa/30-60 sec, 552 MPa/up to 180 sec) demonstrated 100% killing effect on nematode worms such as *Anisakis simplex*, but showed such significant effects on color (increased whiteness) and overall appearance as to limit the application of HPP in processing the raw fish (Dong et al., 2003).

Histochemical and morphological changes were observed in *Trichinella spiralis* larvae cells with consequent loss of their infectivity, after exposure to high hydrostatic pressure treatment of 300 MPa (Ohnishi et al., 1994).

Slifko et al. (2000) reported the application of HHP (high hydrostatic processing) at 80,000 psi (550 MPa) for 60 sec and longer was capable of inactivating more than 99.995% of *C. parvum* oocysts (suspended in apple and orange juices), demonstrating the efficiency of HHP as an alternative food treatment.

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CHAPTER III

THE EFFECTS OF E-BEAM IRRADIATION AND MICROWAVE ENERGY ON EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*) EXPERIMENTALLY INFECTED WITH *CRYPTOSPORIDIUM PARVUM*¹

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ABSTRACT

Shellfish have been identified as a potential source of *Cryptosporidium* infection for humans. The inactivation of *C. parvum* and other pathogens in raw molluscan shellfish would provide increased food safety for normal and at-risk consumers. The present study identified the efficacy of 2 alternative food-processing treatments, e-beam irradiation and microwave energy, on the viability of *C. parvum* oocysts in Eastern oysters (*Crassostrea virginica*). Oysters were artificially infected with the Beltsville strain of *C. parvum* and subjected to e-beam and microwave treatments. The effects of the treatments were evaluated by inoculation of the processed oyster tissues using the neonatal mice assay. Significant reductions ($P < 0.05$) in infectivity were observed for in-shell and shucked oysters treated with e-beam irradiation doses of 1.0, 1.5 or 2 kGy. A dose of 2 kGy completely terminated *C. parvum* infectivity and did not adversely affect the visual appearance of the oysters. Oyster material treated with microwave time (temperature) exposures of 1 sec (43.2°C), 2 sec (54.0°C), and 3 sec (62.5°C) showed a reduction in *C. parvum* mouse infectivity of 26.7%, 33.3%, and 46.7%, respectively. Microwave energy treatments at 2 sec and 3 sec showed extensive changes in oyster meat texture and color. Thus due to lack of efficacy and unacceptable tissue changes; microwave treatment of oysters is not considered a viable food processing method.

Key words: *Cryptosporidium parvum*, oysters, shellfish, *Crassostrea virginica*, e-beam irradiation, microwave

INTRODUCTION

The protozoan parasites *Cryptosporidium parvum* and *C. hominis* cause diarrheal disease and gastrointestinal illness in humans in the United States and other countries worldwide (Fayer et al., 1990; O'Donoghue, 1995; Morgan-Ryan et al., 2002; Xiao, et al., 2004). *Cryptosporidium* was recognized as a significant contributor to morbidity and mortality in immunocompromised persons, such as AIDS patients (Fayer et al., 1997a). Other vulnerable populations are young children, pregnant women, and patients treated with immunosuppressive drugs (Guerrant, 1997). Public health concerns about *Cryptosporidium* were heightened in the early 1990s due to waterborne-disease outbreaks of cryptosporidiosis that were linked to surface water drinking sources (Kramer et al., 1996). For example, in 1993 in Milwaukee, Wisconsin an outbreak of cryptosporidiosis sickened more than 400,000 people and killed over 100 people (MacKenzie et al., 1994; Rose, 1997). Surveys have found widespread distribution of *Cryptosporidium* in surface waters in the U.S. and Canada (Rose et al. 1997; LeChevallier et al., 1991; Stone, 1994). *Cryptosporidium* is considered the most important biological water contaminant in the U.S., and has caused massive waterborne epidemics around the world (Fayer et al., 1997a; Fricker et al., 1998; McLauchlin et al., 2000; Moore, et al., 1993) Cryptosporidiosis outbreaks associated with the consumption of food were first reported from freshly pressed (unpasteurized) apple cider (Millard et al., 1994), unpasteurized apple juice (Anon., 1996b), and chicken salad (Centers for Disease Control and Prevention, 1986). Outbreaks associated with consumption of raw and undercooked meat products were due to faecal transmission of *Cryptosporidium* from cattle to the surface of carcass during dressing (McEvoy et al., 2003).

Major sources for *Cryptosporidium* contamination are wastewater treatment facilities and animal farms, especially ruminant farms (Fayer et al., 1997b; Graczyk, 1997; Lindsay et al., 2000; Thompson, 2003). Rainfall, floods, and sewage overflows wash *Cryptosporidium* oocysts into rivers, bays, and oceans, and as a result, contaminate fresh and marine waters with *Cryptosporidium* oocysts. This contamination can find its way into some aquatic organisms, such as shellfish, which become part of the human food supply.

Researchers detected *C. parvum* oocysts in shellfish worldwide (Chalmers et al., 1997; Fayer et al., 1998; Freire-Santos et al., 2000; Gomez-Bautista et al., 2000). All commercial oyster-harvesting sites tested in Maryland tributaries to Chesapeake Bay (Fayer et al., 1998; Graczyk et al., 2000) were found contaminated. Because oysters are filter feeders and are often

consumed raw, the researchers concluded that *C. parvum* oocysts in oysters pose a potential public health problem and possible source for foodborne-disease. A more recent and geographically wider survey found *Cryptosporidium* species in commercial shellfish (oysters and/or clams) from 64.9% of sites sampled in 13 Atlantic coast states from Maine to Florida and New Brunswick, Canada (Fayer et al., 2003). Reports of food related outbreaks of this emerging disease are difficult to document and most likely under-reported (Fayer et al., 2000).

Customary processing operations may not completely eliminate *C. parvum* oocysts from the gills and other parts of the oysters. The parasite is able to survive in the tissue of fresh shellfish from harvest through consumption and is resistant to treatment with many toxic chemicals (liquids and gasses) and can survive several months at refrigeration temperatures.

The objectives of this study were to identify the effects of two alternative commercial food processing operations (e-beam irradiation and microwave energy treatments) on the viability of *C. parvum* oocysts in Eastern oysters using the neonatal mouse model to test for oocyst infectivity (Lindsay et al., 1999).

MATERIALS AND METHODS

Oocysts

Cryptosporidium parvum oocysts (Beltsville isolate) were collected and concentrated from the feces of experimentally infected dairy calves (Fayer and Ellis, 1993) at the Environmental Microbial Safety Laboratory, U.S. Department of Agriculture, Beltsville, Maryland. Purified oocysts were shipped on cold packs to the Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA. Oocysts were stored at $4^{\circ}\text{C} \pm 0.01^{\circ}\text{C}$ and were less than 2 months old when used for the experiment.

Infection and sampling of oysters

Eastern oysters (*Crassostrea virginica*) were obtained from a commercial source (Cowart Seafood Corp., Lottsburg, VA) and used in this study. Oysters (12 to 15/10 gallon aquaria) were acclimated for 2 weeks in artificial sea water (salinity, 12-15 ppt). 11-15 oysters per 10-gallon tank were held at aquatic facilities (Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA). The oysters were alive before inoculation with *C. parvum* and then prepared after 24h PI (post inoculation) for e-beam irradiation treatment and microwave energy exposure.

The pH and water temperature were checked daily and maintained between pH 8.0 and pH 8.2, and 16-19°C. Oysters were fed 2 times per week with “Instant Algae” (3.1 billion cell/ml, Reed Mariculture/Instant Algae Products, San Jose, CA). The aquaria filters were removed for 24 hr to prevent possible entrapment of oocysts in the filter material during the inoculation period but the aeration system (air stones) was functioning constantly during each experiment. A dose of 2×10^7 *C. parvum* oocysts was added to each experimental tank to infect oysters prior to the treatments: under e-beam irradiation and microwave energy.

E-beam irradiation

Oysters were removed from aquaria 24 hours post-inoculation (PI) and shipped overnight on cool packs to a commercial source for e-beam irradiation (SureBeam Corporation, Glendale Heights, IL). Commercial e-beam irradiation treatment was applied to in-shell (36) and shucked (36) oysters at doses of 0 kGy (control), 1.0 kGy, 1.5 kGy, and 2.0 kGy (Table 1). Each treatment was replicated three times.

Microwave irradiation (energy)

Oysters were removed from aquaria 24 hours PI and used for microwave treatment. Infected oysters (n = 40) were shucked and exposed to microwave energy (Amana Commercial Radarange Microwave Oven, Model RC20SE [designed to deliver 2100 watts of microwave power to the oven cavity with frequency of 915 MHz], Amana Refrigeration, Inc., Amana, IA). Microwave treatment exposures were 0 sec (control)/22.4° C; 1 sec/43.2°C; 2 sec/54.0°C; and 3 sec/62.9°C (Table 2). Each treatment was replicated three times.

Processing of infected oysters

Oysters were individually washed in tap water to remove surface contaminating oocysts. Oysters were processed using the general methods described by Fayer et al., 1998. Hemolymph, gill washes and oyster tissues were processed separately and then recombined before examination for *C. parvum* oocysts using a commercial immunofluorescent antibody (IFA) test kit (MERIFLUOR, Meridian Diagnostic, Cincinnati, OH) (Fayer et al., 1998). Briefly, hemolymph was collected by drilling a hole in the anterior part of the upper valve and inserting an 18-gauge needle (fitted to 5 ml syringe) into the adductor muscle to aspirating the hemolymph (for each subjected oyster, needle and syringe used individually). The gills were removed and placed in a 15 ml centrifuge tube with 5 ml of phosphate-buffered saline (PBS) and were vortexed for 15 sec. After centrifuging (3,000 x g) the oyster material for 10 minutes, the resuspended pellets were placed on three-circle wells glass to dry for IFA test. Washes from oyster gills and collected hemolymph were prepared separately before IFA test. The oyster tissue was homogenized in a Stomacher and filtered several times through cheesecloth to remove particulate material. Suspended pellets after centrifuging were pipetted into three circular wells on glass (teflon-coated) microscope slides. For fluorescence microscopy, aliquots (200 µl) of a mixture containing equal parts of hemolymph, gill washings and oyster tissue homogenate, were dried on those 3-welled microscope slides. The slides were processed using the IFA test kit and examined for the presence or absence of oocysts using an epifluorescence microscope (Olympus, BX60 microscope). The IFA test was applied twice for each experimental treatments: before the intubation of mice for mouse bioinfectivity assay to have information on amount of oocysts concentrated in subjected to the treatment oysters, and after mouse infectivity assay to evaluate the effects of those treatments (the presence and absence of *C. parvum* oocysts in intestine of neonatal mice).

Mouse infectivity assay

Infectivity of the processed oyster material (hemolymph, gill washings and oyster tissue homogenate) was examined using the neonatal mice bioassay. Briefly, 5-day-old suckling mice, CD-1, total two hundred forty for e-beam irradiation and one hundred twenty one for microwave energy treatments, were fed 100 μ l/mouse of test material (from three pooled aliquots of 3-4 oysters) by oral gavage. Dose ranged from 7×10^2 to 18×10^2 of *C. parvum* oocysts per tested mouse. Each litter had 9-11 mice, Table 1 and Table 2. All mice in each litter were killed 5 days PI by decapitation. The intestinal tissue (duodenum, jejunum, ileum, cecum, proximal and distal colon) was removed from each mouse pup and placed separately (representing each individual mouse intestine tissue sample) from each mouse in a 15 ml centrifuged tube with 9.0 ml Hank's balanced salt solution containing 1,000 U/ml penicillin and 1,000 μ g/ml streptomycin (pH 7.2), and kept at $4^\circ\text{C} \pm 0.01^\circ\text{C}$ under refrigeration. Intestines were homogenized using a Stomacher. The centrifuged homogenate (3,000 x g) of this tissue of each mouse (200 μ l on triplicate wells) was examined for *C. parvum* using the IFA test kit.

Statistical analysis

Significant differences were determined with PROC GLM of SAS statistical software (SAS Institute, Inc., Cary, NC). When significant effects were found to be present, pairwise comparisons were performed using Bonferroni's *t* test on adjusted mean inactivation (reduction) levels generated by generalized linear model.

RESULTS

Effects of e-beam irradiation on *C. parvum* infectivity

E-beam irradiation of in-shell and shucked oysters at all dose levels was significant ($P < 0.05$) in reducing infectivity of *C. parvum* for neonatal mice (Table 1). Complete inactivation of *C. parvum* infectivity was achieved at 2 kGy. No visual changes were observed in oyster tissues after e-beam treatments.

Effects of microwave treatment on *C. parvum* infectivity

Microwave irradiation of in-shell and shucked oysters was not effective in significantly ($P > 0.05$) reducing the infectivity of *C. parvum* in oysters for nursing mice (Table 2). Additionally, microwave treatment showed extensive changes in meat texture and color changes starting at 2 sec (54.0°C) to 3 sec (62.5°C) indicating cooking had occurred.

DISCUSSION

Microwave and ionizing irradiation (gamma, x-ray or e-beam) are two treatments that have been investigated to control infectivity of food-borne bacteria, viruses, and parasites in various foods, including some seafood. Most of the irradiation research has used gamma radiation. Research indicates that high-energy ionization, whether produced by gamma, x-ray or e-beam radiation, has generally similar chemical and biological effects (Wilkinson and Gould, 1998). Kilgen (2001) summarized research on the effects of low, medium and high doses of ionizing radiation on fresh, frozen, and processed seafood products. Kilgen (2001) noted that, 1) in general, Gram-negative bacteria are more sensitive to ionizing radiation than Gram-positive bacteria, and 2) a dose of 4 kGy was found sufficient to eliminate non-spore-forming pathogens in many kinds of foods, including frozen seafood. In regard to oysters, Kilgen (2001) summarized studies and reported that radiation doses of 1.0 to 1.5 kGy are necessary to reduce bacterial numbers to a non-detectable level.

These studies found that level of 1.5 kGy to eliminate *Escherichia coli*; 1.2 kGy to eliminate *Vibrio* spp.; 1.5 kGy to eliminate *V. vulnificus*; 1.0 kGy to eliminate *V. cholerae*; 1.0 kGy to eliminate *V. parahaemolyticus* seeded in *C. virginica*. Alternative food processing treatments may be implemented in seafood processing if they provide a 5-log reduction of the pertinent pathogen. Tauxe (2001) reports that elimination of 99.999% of bacteria (a so-called 5-logarithm kill) takes 5 times the irradiation dose needed for a 1 log kill and would reduce a million bacteria to ten. For example, to reduce *Campylobacter* in meat it takes 0.2 kGy with one decimal log or 1 kGy to reduce it by 5 decimal logs. Gamma irradiation has been recognized as a most effective preservation technique for several decades and has been used to extend food shelflife and to assure the safety of food supplies (Farcas et al., 1997).

Jenkins et al. (1995) showed that gamma irradiated and nonirradiated *Eimeria tenella* sporozoites exhibited differential uracil uptake and expression of 7- to 10-kDa metabolic antigen. Metabolic 7- to 10-kDa antigen expression by 25 kRad (2.5 kGay) irradiated sporozoites was greatly reduced compared to non-irradiated or 15 kRad (1.5 kGay) irradiated intracellular sporozoites. The findings suggested that the metabolic 7- to 10-kDa antigen plays a role in protective immunity elicited by non-irradiated or 15 kRad (1.5 kGay) irradiated *E. tenella* sporozoites. Our present study on e-beam irradiation treatment supports the need for more sensitive detection methods (e.g., PCR-based analysis), as also noted by Jenkins et al. (1995).

The effects of gamma radiation with ^{137}Cs at 0.4 to 0.8 kGy were tested on unsporulated and sporulated *Toxoplasma gondii* oocysts (Dubey et al., 1998). Results showed that irradiation at 0.5 kGy are effective in “killing” of these coccidian oocysts on fruits and vegetables. The results were used to develop a model system for sterilization of fruit contaminated with other coccidia such as *Cyclospora* or *Cryptosporidium*. In contrast, the present study uses e-beam rather than gamma radiation, and uses higher irradiation levels (1.0, 1.5, and 2.0 kGy).

E-beam electrons have a limited penetration depth of about 5 cm or less as compared to x-rays that have significantly higher penetration depth (60 - 400 cm) depending upon the energy used. However, this limited penetration fits the size of oysters and is suitable for treating oysters. The present study indicates that oysters received a uniform distribution of e-beam penetration sufficient to kill *C. parvum* oocysts. The results of this study indicate that irradiation doses equal to, or less than, 2.0 kGy may serve in a commercial process to eliminate the enteric protozoan pathogen *C. parvum* in fresh oysters, shucked or in shell. Because the U.S. Food and Drug Administration (FDA) has received a request from the National Fisheries Institute Inc. to allow irradiation of shellfish at a maximum of 2.0 kGy, it appears that irradiation up to 2 kGy would be in the range of acceptable quality for fresh oysters.

In contrast to ionizing radiation, the microwave produces nonionizing radiation and is too weak to break chemical bonds. The microwave radiation is absorbed at the molecular level, and results in heating and vibration changes of the molecules. Microwave heating has been used to destroy microorganisms in food, but there is a long-standing debate about the thermal and non-thermal effects produced by microwave treatment of food (Anantheswaran and Ramaswamy, 2001). In a recent review of this controversy concerning microwave biological effects produced by non-thermal effects as well as thermal effects, Banik et al. (2003) provided examples of recent research that claims to show non-thermal effects. From these recent studies, microwave does indeed athermally induce various physiological effects. However, the research results do not always specify the mechanism to account for the non-thermal effects. A study of the effects of microwaves on developmental and infective stages of three parasites (*Eimeria nieschulzi*, *Strongyloides ratti*, and *Taenia taeniaeformis*) showed that microwave radiation was extremely effective in killing or preventing development of helminth and protozoan parasites without unduly distorting eggs or developmental stages (Conder and Williams, 1983).

Bouchet and Boulard (1991) studied the effect of microwaves on the ultrastructure of oocysts of *Eimeria magna*. The oocysts were exposed to microwave energy (2,450 MHz; 600 W) for different durations (10, 15 and 20 sec). The microwave treatment progressively destroyed the three layers of the oocyst wall and damaged internal structures. Adams et al. (1999) studied the relationship between temperature and survival of nematodes of the species *Anisakis simplex* in microwave-processed arrow-tooth flounder (*Atheresthes stomias*). Fillets, 14 cm long, 4.5 cm wide, and approximately 1.75 cm high, were inoculated with 10 live nematodes and then microwaved. No viable nematodes survived microwave processing at 160° F (71°C) or 170 °F (77°C). Smaller fillets required only 150° F (65°C) probably due to fewer cold spots during microwave processing. In contrast, larger whole fillets required 170° F (77°C). Adams et al. (1999) indicated that damage to the nematode's cuticles was apparent after treatment. Inactivation of the parasites was attributed to the thermal effects of the microwave treatment (Adams et al., 1999). Many factors influence microwave thermal processes (mass of objects, shape of objects, specific heat, thermal conductivity, etc.) that are the same as conventional heating (Heddleson and Doores, 1994).

Microwave treatment reduced the infectivity of *C. parvum* in oysters but did not completely prevent infectivity at the levels tested (Table 2). The oyster tissues appeared cooked at treatment time/temperature of 2 sec (54.0°C) or 3 sec (62.5°C). According to the U.S. Food and Drug administration (FDA) oysters exposed to temperatures exceeding 120° F (49°C) (temperature at which the gills curl) cannot be sold as raw oysters. Therefore, microwave energy treatment at energies that render *C. parvum* noninfectious would render oysters unsuitable for marketing as a raw product.

CONCLUSIONS

The results of this study indicate that e-beam irradiation treatment is capable of eliminating enteric protozoan pathogen *C. parvum* in fresh oysters, whereas microwave treatment at levels that eliminate this pathogen render oysters unsuitable for sale as raw product. E-beam irradiation treatment at 2.0 kGy produced 100% reduction in *C. parvum* oocyst viability.

As a method to detect *Cryptosporidium* oocyst viability in oysters after e-beam and microwave treatment, this study used a mouse bioassay with IFA oocyst identification.

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TABLES

Table 1. Effects of various doses of e-beam irradiation treatment on *Cryptosporidium parvum* infectivity in Eastern oysters (*Crassostrea virginica*) using the neonatal mouse bioassay.

Dose e-beam irradiation	Total No. oysters	No. positive/ total no. ¹	% reduction ²
<hr/>			
Unshucked oysters	36		
Control ³		28/30	6.6 a ⁴
1.0 kGy		13/30	56.7 b
1.5 kGy		11/30	63.3 bc
2.0 kGy		0/30	100.0 c
<hr/>			
Shucked oysters	36		
Control		30/30	0.0 a
1.0 kGy		16/30	46.7 b
1.5 kGy		8/30	73.3 bc
2.0 kGy		0/30	100.0 c

¹ Number of animals positive for *C. parvum*/total number of animals tested.

² Percentage of reduction is average means from three replications per each dose of irradiation.

³ The control is untreated artificially infected oysters with *C. parvum*.

⁴ Treatments with the same letters are not significantly different.

($p < 0.05$).

Table 2. Effects of various temperatures of microwave treatment on *Cryptosporidium parvum* infectivity in Eastern oysters (*Crassostrea virginica*) based on the neonatal mouse bioassay.

Dose microwave time (sec)	Temperature °F/°C		No. positive/ total ¹	% reduction ²
	Initial (±SD) ⁴	Terminal (±SD) ⁴		
Control ³	72.4/22.4(0.20)	the same	30/30	0.0 a ⁵
1 sec	72.2/22.3(0.12)	109.7/43.15(0.18)	22/31	26.7a
2 sec	72.3/22.4(0.85)	129.3/54.0 (1.70)	20/30	33.3a
3 sec	72.2/22.3(0.10)	145.3/62.9 (0.16)	16/30	46.7a

¹ Number of animals positive for *C. parvum*/total number of animals tested.

² Percentage of reduction is average means for three experiments.

³ The control is untreated artificially infected oysters with *C. parvum*.

⁴ Resulted mean of temperatures of experimental oysters during the microwave time settings. measured in °F/°C conversion for three replications and standard deviation.

⁵ Treatments with the same letters are not significantly different. (p < 0.05).

CHAPTER IV

THE EFFECT OF HIGH HYDROSTATIC PRESSURE TREATMENT ON EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*) EXPERIMENTALLY INFECTED WITH *CRYPTOSPORIDIUM PARVUM*¹

¹To be submitted to the Journal of Parasitology

ABSTRACT

Shellfish have been identified as a potential source of *Cryptosporidium* infection for humans. The inactivation of *C. parvum* and other pathogens in raw molluscan shellfish would provide increased food safety for normal and at-risk consumers. The present study identified the efficacy of a non-thermal alternative food processing treatment, high hydrostatic pressure (HHP), on the viability of *C. parvum* oocysts in Eastern oysters (*Crassostrea virginica*). Oysters were artificially infected with the Beltsville strain of *C. parvum* and subjected to high pressure processing treatments. The effects of the treatments were evaluated by inoculation of the processed oyster tissues using the neonatal mice bioassay. High pressure processing of shucked Eastern oysters at all pressures tested (305, 370, 400, 480, and 550 megaPascals [MPa]) was significantly effective ($P < 0.05$) in reducing infectivity of *C. parvum* oocysts. A dose of 550 MPa at 180 sec of holding time produced the maximum decrease of infectivity (93.3%).

Measurement of tristimulus color values of pressurized raw oysters at extended processing times from 120sec to 360sec at 550 MPa showed a small increase in whiteness of oyster meat. This non-thermal processing treatment shows promise for commercial applications to improve safety of seafood and reduce public health risks from cryptosporidiosis.

Key words: *Cryptosporidium parvum*, oysters, shellfish, *Crassostrea virginica*, High Pressure Processing.

INTRODUCTION

Foodborne disease outbreaks associated with the protozoan parasite *Cryptosporidium* are an emerging public health concern. Since 1993 several foodborne outbreaks of cryptosporidiosis have shown the threat from *Cryptosporidium* is not limited to waterborne outbreaks (Millard et al., 1994; Rose and Slifko, 1999; Slifko et al., 2000). Cryptosporidiosis has caused massive waterborne epidemics around the world (Fayer et al., 1997; Fricker et al., 1998; McLauchlin et al., 2000; Moore, et al., 1993; Rose et al., 1997).

Concerns about *Cryptosporidium* first arose due to waterborne outbreaks of cryptosporidiosis, including a 1993 outbreak that affected more than 400,000 people and killed over 100 people in Milwaukee, Wisconsin (MacKenzie et al., 1994; Kramer et al. 1996; Rose, 1997). Cryptosporidiosis is a cause of gastrointestinal illness in humans and domestic animals worldwide (Fayer et al., 1990; O'Donoghue, 1995; Olson et al., 1999; Morgan-Ryan et al., 2002; Xiao, et al., 2004) and is a significant contributor to mortality of immunocompromised or immunosuppressed persons, such as AIDS patients (Fayer et al., 1997). Cryptosporidiosis has caused massive waterborne epidemics around the world (Rose et al., 1997).

Cryptosporidium is widely distributed in the surface waters worldwide (LeChevallier et al., 1991; Levine and Craun, 1990). Significant sources of *Cryptosporidium* include wastewater treatment facilities and animal farms, especially ruminant farms (Fayer et al., 1997; Graczyk, 1997; Rose et al., 1997). Rainfall, floods, and sewage overflows wash *Cryptosporidium* oocysts into rivers, bays, and oceans and contaminate fresh and marine waters with *Cryptosporidium* oocysts. The Chesapeake Bay is a prominent example of susceptibility to environmental contamination with *Cryptosporidium parvum* (Fayer et al., 1998; Graczyk et al., 2000). As a result, *Cryptosporidium*-contaminated water can be introduced into the food supply in various ways, such as water used in food preparation or processing (e.g. fruit juices), or as water ingested or filtered by organisms (e.g. shellfish).

Researchers detected *Cryptosporidium parvum* oocysts in Eastern oysters (*Crassostrea virginica*) at all commercial oyster harvesting sites tested in Maryland tributaries to Chesapeake Bay (Fayer et al., 1998; Graczyk et al., 2000). Because oysters are filter feeders and are often consumed raw, the researchers concluded that *C. parvum* oocysts in oysters pose a potential public health problem as a possible source for the foodborne-disease of cryptosporidiosis. Additional research found *Cryptosporidium* species in commercial shellfish (oysters and/or

clams) from 64.9% of sites sampled in 13 Atlantic coast states from Maine to Florida and New Brunswick, Canada (Fayer et al., 2003).

The development of food processing treatments to reduce or eliminate *C. parvum* oocysts in oysters could help protect shellfish consumers from this emerging pathogen. High pressure processing (HPP) is a treatment that has been used to control infectivity of foodborne pathogens in various foods (Tewari et al., 1999). High pressure processing is especially suited for use with fish and shellfish because it provides non-thermal treatment of food pathogens and longer shelflife while preserving the sensory qualities of the seafood (Flick, 2003).

High pressure processing inactivated *Vibrio parahaemolyticus* and *Vibrio vulnificus* in pure cultures in Eastern oysters (Koo et al., 2002; Cook, 2003). Berlin et al. 1999 reported that *Vibrio* species were susceptible to HPP treatment at pressure levels from 200 to 300 MPa (1 MPa = 10 atm = 147 psi).

This food processing operation, HPP, reduced number of microorganisms in Pacific oysters (Shiu and Morrissey, 1999; Calik et al., 2002) and also provided shucking process for the oysters subjected to that treatment (He et al., 2002). Different picornaviruses have various inactivation responses to HPP. For example, exposure of coxsackievirus at 400, 500, and 600 MPa for 5 min, resulted in 3.4-, 6.5-, and 7.6-log tissue culture infection dose (50%) (TCID₅₀), and human parechovirus-1 at the same doses and duration, demonstrated 1.3-, 4.3-, and 4.6-log TCID₅₀, as a reduction, respectively (Kingsley et al., 2004). High pressure processing inactivated the parasite *Anisakis simplex* in finfish at 200 MPa for 10 min at a temperature between 0 and 15°C (Molina-Garcia and Sanz, 2002; Dong et al., 2003). Dose of HPP at 550 MPa was applied to inactivate *C. parvum* oocysts in apple and orange juice (Slifko et al., 2000).

The objective of this study was to identify the effects of high pressure processing on the viability of *C. parvum* oocysts in Eastern oysters (*Crassostrea virginica*) using the neonatal mouse bioassay (Lindsay et al., 1999).

MATERIALS AND METHODS

Oocysts

Cryptosporidium parvum oocysts (Beltsville isolate) were collected and concentrated from the feces of experimentally infected dairy calves at the Environmental Microbial Safety Laboratory, U.S. Department of Agriculture, Beltsville, MD (Fayer and Ellis, 1993). Purified oocysts were shipped on cold packs to the Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA. Oocysts were stored at $4^{\circ}\text{C} \pm 0.01^{\circ}\text{C}$ and had less than 2 months old when used.

Infection and sampling of oysters

A total of 183 Eastern oysters were obtained from a commercial source (Cowart Seafood Corp., Lottsburg, VA) and used in this study. Oysters (12 to 15/aquarium) were acclimated in artificially sea water (salinity, 12-15 ppt) for 2 weeks. The pH and water temperature were checked daily and maintained between pH 8.0 and pH 8.2, and 16-19° C. Oysters were fed 2 times with “Instant Algae” (3.1 billion cell/ml, Reed Mariculture/Instant Algae Products, San Jose, CA). 11-15 oysters per 10-gallon tank were held at aquatic facilities (Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA). The oysters were maintained alive before inoculation with *C. parvum* and then prepared after 24h PI for HPP

The aquaria filters were removed for 24 hr to prevent possible entrapment of oocysts in the filter material during the inoculation period but the aeration system was functioning constantly during each experiment. A dose of 2×10^7 *C. parvum* oocysts was added to each 10-gallon experimental tank.

HHP treatment

Oysters were removed from aquaria 24 hours post-inoculation (PI) and subjected to HHP treatment. The hemolymph was briefly collected by drilling a hole in the anterior part of the upper valve and inserting an 18-gauge needle (fitted to 5 ml syringe) into the adductor muscle to aspirating the hemolymph (for each subjected oyster, needle and syringe used individually). The

hemolymph from infected oysters was taken separately and placed with the rest of the oyster tissue in vacuum-sealed plastic double bags. Infected oysters were shucked before the HPP treatment exposure. The bags were placed into a high pressure-processing (HPP) unit (Quintus Food Press QFP 35L-600 Model, Flow International Corporation, Avure Technologies, Kent, WA) with 7XS-6000-intensifier pump and maximum operating pressure of 600 MPa (87,000 psi). High pressure processing unit was installed and operated at Virginia Tech's Department of Food Science and Technology. High hydrostatic pressure was applied on shucked (n=278) oysters at 305 MPa (45,000 psi); 370 MPa (55,000 psi); 400 MPa (60,000 psi); 480 MPa (70,000 psi); and 550 MPa (80,000 psi) with holding durations of 0 sec, 60 sec, 120 sec, and 180 sec. In addition, a HHP treatment of 80,000 psi (550 MPa) had additional holding times of 0 sec, 120 sec, 240 sec, and 360 sec. Each HPP treatment was replicated three times with two replication trials for the extended duration at 550 MPa (Table 1 and Table 2).

Processing of infected oysters

Oysters were individually washed in tap water to remove surface contaminating oocysts. Oysters were processed using the methods described by Fayer et al. (1998). Hemolymph was collected and then the gills were removed and placed in a capped 15 ml centrifuge tube with 5 ml of phosphate-buffered saline (PBS) and vortexed for 15 sec. After centrifuging (3,000 x g) for 10 min, washes from gills and collected hemolymph were prepared separately. Each individual oyster was homogenized in a stomacher machine for 5 min. The resulting homogenate was filtered several times through cheesecloth, and pelleted. The remaining oyster tissue was homogenized in a Stomacher, and filtered several times through cheesecloth to remove particulate material. Suspended pellets (200 µl from this mixture of three: hemolymph, gill washes, and remain oyster body tissue) were pipetted into three 11.5-mm-diameter circular wells on glass (teflon-coated) microscope slides. To determine whether the inoculated oysters contained infectious *C. parvum* oocysts, the collected hemolymph, gill washings and oyster tissue were combined and analyzed for the presence of *C. parvum* oocysts with immunofluorescent antibody (IFA) using the MERIFLUOR test kit (Meridian Diagnostic, Cincinnati, OH) (Fayer et al., 1998). For fluorescence microscopy, 200 µl of test sample (hemolymph, gill washings and oyster tissue homogenate) was dried on treated 3-welled

microscope slides. The slides were processed using the test kit and examined with an epifluorescence microscope (Olympus, BX60 microscope).

Mouse infectivity assay

Oyster tissues were processed prior to mouse inoculation. The infectivity of the material was examined using the neonatal mouse bioassay (Lindsay et al., 1999). Briefly, Briefly, 5-day-old suckling mice, CD-1, total of six hundred eighty three for high hydrostatic treatment at all doses of exposure, were fed 100 μ l/mouse of test material (from three pooled aliquots of 3-4 oysters/per litter) by oral gavage. Dose ranged from 9×10^2 to 17×10^2 of *C. parvum* oocysts per tested mouse. Each litter (9-11 mice, Table 1 and Table 2) represented a separate treatment at each pressurization exposure. All mice in each litter were killed 5 days PI by decapitation. The intestinal tissue (duodenum, jejunum, ileum, cecum, proximal and distal colon) was removed from each mouse pup and placed separately (representing each individual mouse intestine tissue sample) from each mouse in a 15 ml centrifuged tube with 9.0 ml Hank's balanced salt solution containing 1,000 U/ml penicillin and 1,000 μ g/ml streptomycin (pH 7.2), and kept at $4^\circ\text{C} \pm 0.01^\circ\text{C}$ under refrigeration. Intestines were homogenized using a Stomacher. The centrifuged homogenate ($3,000 \times g$) of this tissue of each mouse (200 μ l on triplicate wells) was examined for *C. parvum* using the IFA test kit.

Color measurement

Tristimulus color was measured with a colorimeter (Minolta Chroma Meter CR-200, Minolta, Ramsey, NJ) for three samples of oysters per pressure treatment at 550 MPa to evaluate the presence of alterations in a color of experimental oysters during this exposure. Three measurements were taken from random areas of each oyster and averaged ($n = 9$). The **L***-value represented readings from 0 (black color) to +100 (white color), **a***-value indicated green/redness (-80/+100), and **b***-value described the blue/yellow (-80/+70) changes in a color. One-way ANOVA (analysis of variance) and Tukey's HSD test ($P < 0.05$) was applied for statistical analysis of color changes in experimental oysters for extended holding time under the same pressure level (550 MPa).

Statistical analysis

Significant differences were determined with PROC MULTTEST of SAS statistical software (SAS Institute, Inc., Cary, NC). When significant effects were found, Bonferroni's *t* test (multiple comparisons) was performed on adjusted mean inactivation (reduction) levels generated by generalized linear model. Logistic Regression Analysis was used to model the probability of survival of *C. parvum* oocysts in mice to evaluate the pressure/duration (time) relationship.

RESULTS

Effect of high pressure processing on *C. parvum* infectivity

High pressure processing of shucked oysters at all pressures and time tested (305 MPa, 370 MPa, 400 MPa, 480 MPa, 550 MPa) was significantly effective ($P < 0.05$) in reducing infectivity of *C. parvum* oocysts as measured by the neonatal mouse bioassay (Table 1 and Figure 1). The HPP process achieved a maximum reduction of *C. parvum* infectivity of 93.3% at 550 MPa for 180 sec.

The effect of increasing processing times at each pressure had mixed results on reducing infectivity of *C. parvum* oocysts; an increase in processing time from 60 sec to 120 sec to 180 sec did not produce significant reduction in infectivity at all pressures tested. An extended test of processing times at 550 MPa (240 sec, 360 sec) showed a tailing effect in reduction of infectivity (Table 3 and Figure 3). Lost of translucency (from visual observations) in oyster tissue at these parameters of HPP exposure was appeared.

Effect of high pressure processing on color of oyster meat

Table 3 displays the results of increasing treatment times at 550 MPa on tristimulus color values (**L***, **a***, **b***) of raw oysters. Lightness values (**L***) ranged from 60.67 for 0 sec (control oysters) to 73.26 for 360 sec. As treatment times increased from the 120 sec to 360 sec at 550 MPa pressure, a small increase in whiteness of the oyster tissue appeared, as compared to the untreated (control) oysters. No similar trends were presented in **a*** and **b*** values data from the colorimetric test. This effect (whiteness of the oyster tissue) occurred at the processing pressure and time that produced a 93.3% inactivation of *C. parvum* (550 MPa for 180 sec).

DISCUSSION

The results showed that high pressure processing (HPP) of shucked Eastern oysters produced significant reductions in infectivity of *C. parvum* oocysts as measured by the neonatal mice bioassay (Table 1 and Figure 1). The reductions varied from 30% (400 MPa, 180 sec) to 93.3% (550 MPa, 180 sec). The results also showed that an increase in HHP exposure time beyond 180 sec at 550 MPa did not produce increased reductions in infectivity of *C. parvum* oocysts (Table 1 and Figure 1).

Results of the extended test of HPP times at 550 MPa (240 sec, 360 sec) showed a tailing effect on reductions in infectivity of *C. parvum* oocysts (Figure 3 and Table 2). Tailing effects produced by HPP have been reported and analyzed in research on the effects of HPP on microbial inactivation (Barbosa-Canovas and Rodriguez, 2002; Tay et al., 2003).

Overall, the effects of HPP on the inactivation of *C. parvum* oocysts in this study are similar to the effects of HPP on microbial inactivation. Barbosa-Canovas and Rodriguez (2002) reported that an increase in pressure increases microbial inactivation, but increasing the treatment time does not necessarily increase microbial death rates.

The results show that an increase in pressure from 305 MPa to 550 MPa generally increases the percent reduction of *C. parvum* oocysts in oysters. However, the results also show that 400 MPa produced a significant scatter in percent reduction data from the general trend. One possible explanation for this deviation is variations in mice intubation doses. Doses ranged from 9×10^2 to 17×10^2 of *C. parvum* oocysts per tested mouse, from exposed under the HPP contaminated and control groups of oysters. These intubation doses depended on numbers of oocysts accumulated by oysters in the experimental tank. Varied accumulation of *C. parvum* oocysts by oysters occurred in the 24h post-inoculation period. The preparation of oyster tissue inocula for mice bioassay intubation process requires “Stomaching” to separate the oocysts from oyster tissue, followed by immunofluorescent microscopy observation for further detection of *C. parvum* oocysts. Applications of homogenization for separating oocysts from the food matrix limit the application in solid food products (vegetables, meat, shellfish) because of interference from food debris with oocysts leading to an overall reduction in total oocyst recovery (Moriarty et al., 2004).

Another way to view the results of this study is to smooth out the scatter produced by process holding times and combine the results for the three times at each pressure level. The plot

of pressure data after combining the three times for the same pressure level is displayed in Figure 2. The results show that an increase in pressure from 305 MPa to 550 MPa generally increases the percent reduction of *C. parvum* oocysts. Figure 2 also shows the deviation at 400 MPa from the general trend.

Based on the experiment's results, a Logistic Regression Analysis was used to model the probability of survival of *C. parvum* oocysts in mice given information on pressure and processing time. The logistic model fit is the following:

$$p = \frac{e^{-9.1879+0.000353*pressure-3.28E-8*pressure^2}}{1 + e^{-9.1879+0.000353*pressure-3.28E-8*pressure^2}}$$

Because processing time turned out not to be significant in building the model, processing time was not included in the final logistic model. The fitted values from Logistic Regression Analysis are shown in Figure 4.

Appearance of lighter color (whiteness) in pressurized oysters occurred at increased holding times of 2 min to 6 min at 550 MPa. He et al. (2001) reported that HHP between 235-270 MPa with holding time of 1-2 min is effective in shucked oysters and in reducing microbial counts, but caused only minimal sensory changes to the appearance of raw oysters.

A HPP study at lower pressure for shucking oysters at 235-270 MPa with a holding time of 1-2 min reported minimal changes to the appearance of the raw oyster product (He et al., 2001). High pressure processing treatment of turbot (*Scophthalmus maximus*) at 100-200 MPa with treatment times of 15 to 30 minutes produced loss of transparency and a cooked appearance (Chevalier and Ghoul, 2001). These treatments of raw salmon and arrow tooth flounder (207 MPa/180 sec, 276 MPa/90-180 sec, 414 MPa/30-60 sec, 552 MPa/up to 180 sec) demonstrated a 100% killing of nematode worms such as *Anisakis simplex* but showed such significant effects on color (increased whiteness) and overall appearance as to limit the application of HPP in processing the raw fish (Dong et al., 2003). It appears that oysters are more resistant to HPP-induced color changes than fish.

Kingsley et al. (2004) reported that although HPP treatment provides a solution for HAV inactivation for raw shellfish, and possibly other food products, the data presented offers limited function towards inactivation of some picornaviruses. The potential of pressures greater than 600

MPa to inactivate viruses such as AiV, PV-1, and CBV5 has yet to be examined. For future studies, infected oysters with *C. parvum* exposed to the doses higher than 550 MPa of HPP may achieve higher level of inactivation of this pathogen, with results greater than 3-log reduction. Non-thermal processing technology, such as HHP, results in elimination or reduction of microbial and enzymatic spoilage in a fresh fish and shellfish. As a result, HHP contributes to shelflife preservation and plays an important role in the quality of the market product (Ashie et al., 1996).

A five-log (100,000-fold) reduction in pathogenic organisms is required for juice products. An HPP exposure of juice for 45 and 60 sec at 70,000 psi (480 MPa) or 85,000 psi (550 MPa) meet that requirement (Anstine, 2002). A previous study on whole Pacific oysters (*Crassostrea gigas*) using high pressure processing treatment from 207 to 310 MPa at 0, 1, and 2 min and stored at $< 4^{\circ}\text{C}$ with evaluation over 27 days, showed the effectiveness of HPP in shucking and in decreasing the microbial concentrations and prolonging shelflife of the product (He et al., 2002).

In another study that evaluated the effect of HPP on *C. parvum* oocysts, the application of 550 MPa for 30 sec or greater produced $>99.9\%$ inactivation of *C. parvum* oocysts in apple juice and orange juice (Slifko et al., 2000). Additional research would be needed to determine whether HPP treatment of oysters could achieve a $>99.9\%$ inactivation. Because of the tailing effects on percent reduction of infectivity as shown in this study (Figure 3 and Table 2), the achievement of $>99.9\%$ inactivation of *C. parvum* oocysts in oysters would appear more promising from future research on effects of increasing pressure above 550 MPa than from research on effects of increasing treatment times beyond 360 sec at 550 MPa or below.

CONCLUSIONS

High pressure processing of shucked Eastern oysters at all pressures tested (305 MPa, 370 MPa, 400 MPa, 480 MPa, 550 MPa) was significantly effective ($P < 0.05$) in reducing infectivity of *C. parvum* oocysts as measured by neonatal mice bioassay (Table 1 and Figure 1). The high pressure processing used in this study produced a maximum reduction in infectivity of *C. parvum* oocysts of 93.3% at 550 MPa, 180 sec. The results show that HPP can produce significant reductions in infectivity of *C. parvum* oocysts in Eastern oysters. This non-thermal processing treatment shows promise for commercial applications to improve safety of seafood and reduce public health risks from cryptosporidiosis. However, the risk of significant sensory changes in raw oysters is likely to be greater above 550 MPa. Potential achievement of >99.9% inactivation at pressures above 550 MPa may require unacceptable costs in raw oyster quality and process economics. A different strategy for future research achieving >99.9% inactivation of *C. parvum* oocysts in oysters would be to test whether the combination of HPP treatment (at 550 MPa and below) and another non-thermal treatment (such as e-beam radiation) can increase the maximum reduction of infectivity beyond the 93.3% (550 MPa for 180 sec) achieved in this study.

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TABLES

Table 1. Effects of various pressures and processing time of HHP on *Cryptosporidium parvum* infectivity in Eastern oysters (*Crassostrea virginica*) based on neonatal mouse bioassay.

Pressure (MPa)	Time (sec)	No. positive/total no. ¹	% reduction of oocyst viability from mouse bioassay ²
Control ³	0	30/30	0 a ⁴
305	60	18/30	40.0 ab
305	120	14/30	53.3 bc
305	180	16/30	46.7 ac
Control	0	32/32	0 a
370	60	14/30	53.3 b
370	120	16/30	46.7 bc
370	180	13/30	56.7 bc
Control	0	30/30	0 a
400	60	20/30	33.3 b
400	120	16/30	46.7 bc
400	180	21/30	30.0 bc
Control	0	31/31	0 a
480	60	10/30	66.7 b
480	120	9/30	70.0 bc
480	180	13/30	56.7 bc
Control	0	30/30	0 a
550	60	5/30	83.3 b
550	120	4/30	86.7 bc
550	180	2/30	93.3 bc

¹ Number of animals positive for *C. parvum*/total number of mice tested.

² Percentage reductions are average means for three experiments per each dose of HPP.

³ The control is untreated (without HPP treatment) artificially infected oysters with *C. parvum*.

⁴ Treatments with the same letters are not significantly different.

($p < 0.05$).

Table 2. Effects of pressure treated Eastern oysters (*Crassostrea virginica*) under 550MPa with extended holding time based on neonatal mouse bioassay.

Pressure (MPa)	Time (sec)	No. positive/total no. ¹	% reduction of oocyst viability ² from mouse bioassay
Control ³	0	20/20	0 a ⁴
550	120	5/20	75.0 b
550	240	7/20	65.0 bc
550	360	4/20	80.0 bc

¹ Number of animals positive for *C. parvum*/total number of mice tested.

² Percentage reductions are average means for two experiments.

³ The control is untreated (without HPP treatment) artificially infected oysters with *C. parvum*.

⁴ Treatments with the same letters are not significantly different.
(p < 0.05).

Table 3. Tristimulus color values (color changing) of pressure treated Eastern oysters (*Crassostrea virginica*) under 550MPa with extended holding time.

Pressure Applied (MPa)	Holding Time (sec)	L * ²	a * ²	b * ²
Control ¹	0	60.67 (2.89) a ³	0.63 (0.81) a	10.45 (0.84) a
550	60	65.68 (2.83) ab	1.47 (1.15) a	11.14 (1.67) a
550	120	69.03 (2.74) b	1.37 (0.34) a	12.59 (0.66) a
550	180	69.20 (4.37) b	2.23 (0.62) a	13.20 (1.30) a
550	240	72.44 (1.08) b	2.26 (0.13) a	12.32 (0.46) a
550	360	73.26 (1.30) b	0.31 (0.10) a	10.15 (0.95) a

¹ The control is untreated oysters (without the HHP treatment).

² The L *, a * and b *-values are represented the mean of at least nine measurements from three replication trials on shucked oysters with standard deviations.

³ Treatments with the same letters are not significantly different. (p < 0.05).

FIGURES

Figure 1. Influence of Treatment (HHP) on Reduction in Infectivity of *C. parvum* Oocysts in the Neonatal Mouse Model System.

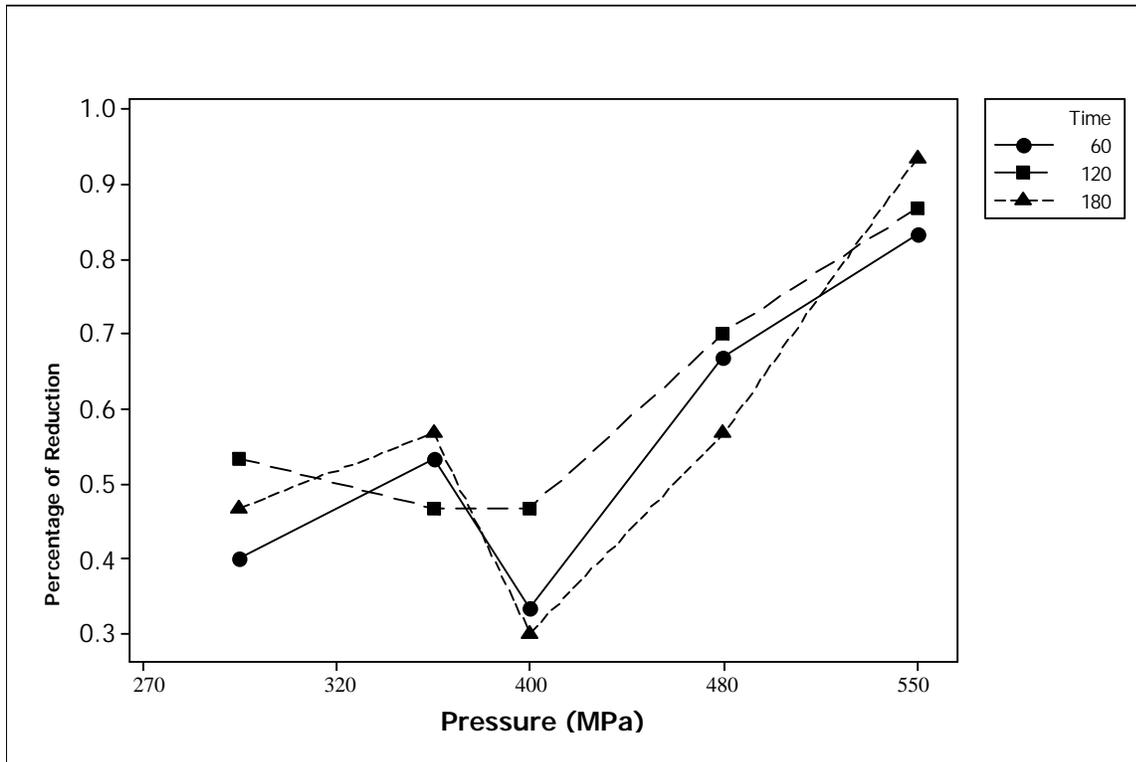


Figure 2. Plot of pressure data (percentage of reduction of *C. parvum* oocyst viability in neonatal mouse model system) after combining three times for the same pressure level.

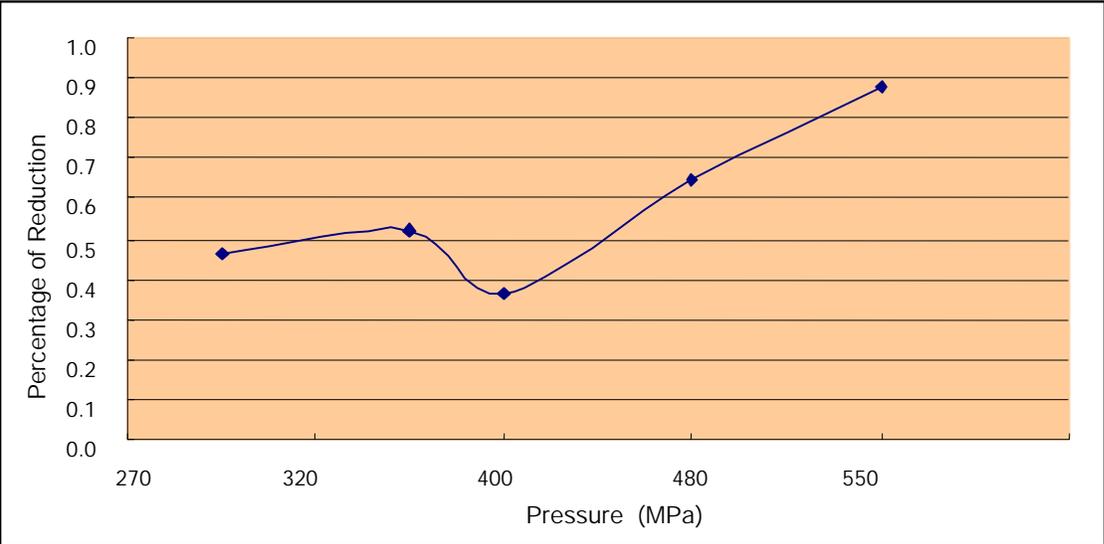


Figure 3. Influence of Treatment (HHP) with extended time under 550 MPa on Reduction in Infectivity of *C. parvum* Oocysts in the Neonatal Mouse Model System.

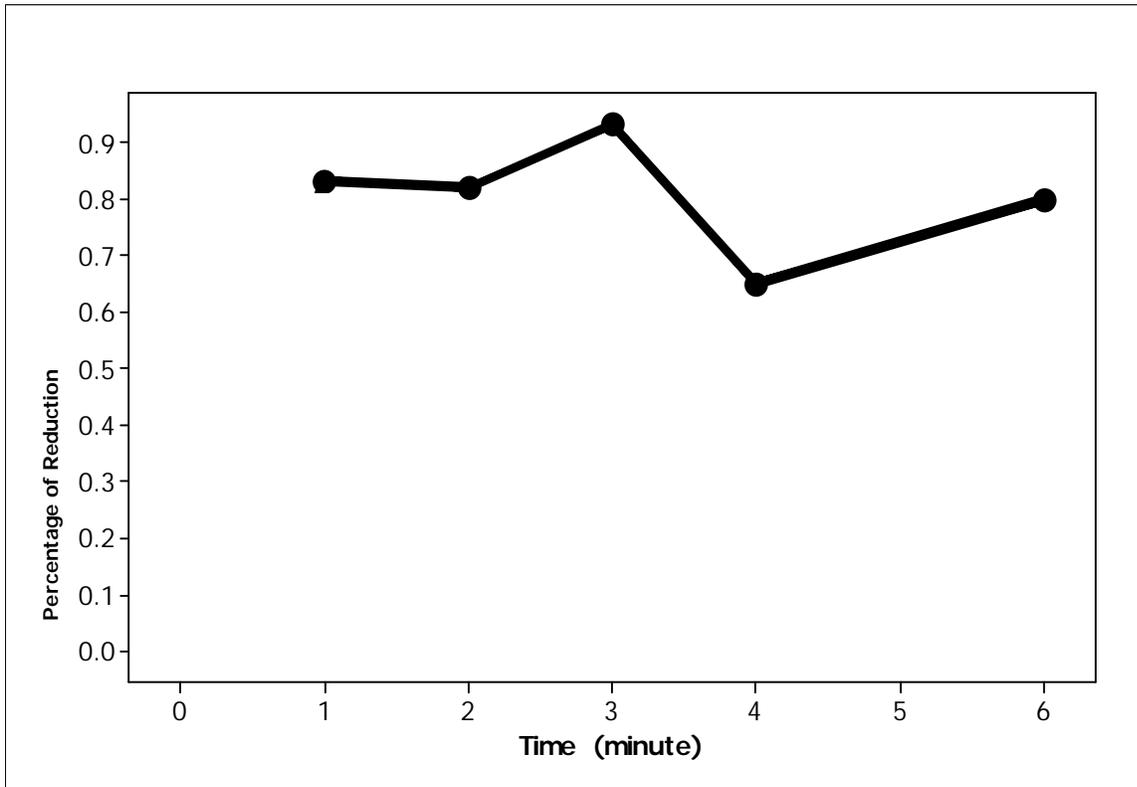
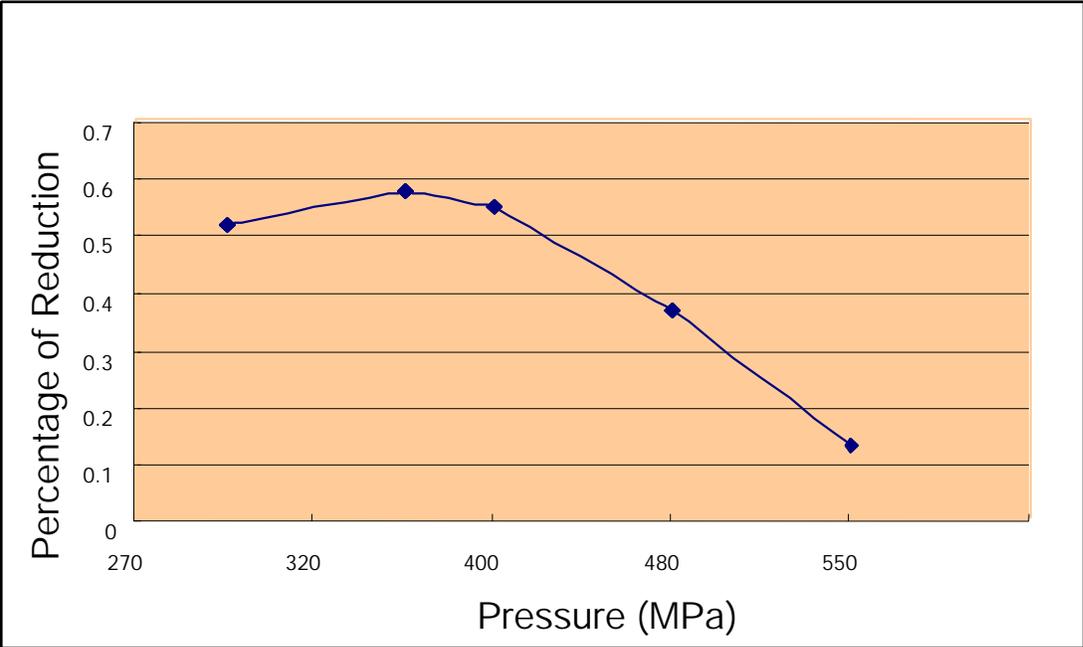


Figure 4. Fitted value (reduction in recovery of viable *C. parvum* oocysts after applied HHP treatment in neonatal mouse model system) from Logistic Regression.



APPENDIX

Table 1. Protocol of e-beam irradiation dosimetry report on dose parameters exposed to infected Eastern oysters (*Crassostrea virginica*) with *C. parvum*.

Product	Dose (kGy)	PCN number	Beam mode	Speed	Monitoring position	Correlated (kGy)	
						Dmin	Dmax
unshucked oysters (3)	1.0	20031080	dual	60	phantom 1	0.6	0.9
					phantom 2	0.6	1.0
unshucked oysters (3)	1.5	20031081	dual	40	phantom 1	0.9	1.4
					phantom 2	0.9	1.5
unshucked oysters (3)	2.0	20031082	dual	29.8	phantom 1	1.3	2.0
					phantom 2	1.3	2.0
shucked oysters (3)	1.0	20031085	lower	40.4	phantom 1	0.9	1.0
					phantom 2	0.9	1.0
shucked oysters (3)	1.5	20031084	lower	26.8	phantom 1	1.4	1.5
					phantom 2	1.4	1.5
shucked oysters (3)	2.0	20020530	lower	20.3	phantom 1	1.9	2.2
					phantom 2	1.9	2.1

Table 2. Protocol of HHP treatment parameters applied on infected Eastern oysters (*Crassostrea virginica*) with *C. parvum*.

Pressure (MPa)	Holding time (sec)	Temperature (°C)				
		Product	Water tank	Vessel	Adiabatic rise	Final
305	60	22.0	24.4	22.7	7.3	30.0
305	120	22.0	24.4	22.4	5.0	29.4
305	180	22.0	24.4	22.7	7.8	30.5
370	60	22.2	26.0	22.7	7.8	30.5
370	120	22.2	26.0	22.2	9.4	31.6
370	180	22.2	26.0	22.7	7.8	30.5
400	60	22.1	21.1	19.0	9.0	28.3
400	120	22.1	21.1	18.7	9.0	27.7
400	180	22.1	21.1	19.0	8.2	27.2
480	60	22.0	22.1	18.7	10.1	28.8
480	120	22.0	22.1	18.7	10.1	28.8
480	180	22.0	22.1	19.0	9.8	28.8
550	60	22.0	22.1	18.7	11.8	30.5
550	120	22.0	22.1	18.7	11.8	30.5
550	180	22.0	22.1	18.9	11.6	30.5
550	240	22.1	22.1	19.0	11.8	30.8
550	360	22.1	22.1	19.4	11.3	30.7

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International Rescue Squad that was main emergency response in Kharkov region of Ukraine. Work included delivery and distribution of medical supplies and medications in areas of natural disasters.

Infectious disease physician, 1983 – 1990

Children's Medical Clinic, National South Railway System, Kharkov, Ukraine. Work included emergency room duties, critical care, diagnosis, prescribing medications for patients, and clinical research in such diseases as cholera and yersiniosis.

Physician, 1989

National Railway Rescue Squad for earthquake relief in Armenia and Tajikistan. Prevented and treated abdominal infection diseases (cholera) and diphtheria. Also, worked as volunteer physician in non-profit rescue squad from Kharkov District with rescue mission in Bucharest, Romania.

Physician, 1986

Part of a team of infectious disease physicians for prevention of food poisoning outbreaks and infectious pathology among children affected by nuclear catastrophe in Chernobyl region.

ACADEMIC EXPERIENCE**Lecturer, 09/1985 -12/1990**

Pediatric Scientific Society, Ukrainian National Institute of Medical Study, Kharkov, Ukraine.

Instructor, 1990 -1991

Infectious Disease Course, Nursing College, Kharkov, Ukraine. Fall and Spring Semesters (35 students).

Consultant and teacher, 1990 - 1992

Iridodignostic procedure and Iridology course, Ukrainian National Research Institute of Psychiatry and Neurology, Kharkov, Ukraine.

AFFILIATIONS

National Capital area Chapter Society of Toxicology, 2002 – present

Institute of Food Technologists, 2001 – present

International Association for Food Protection, 2000 – present

National Ukrainian Pediatric Society, 1983 – 1990

CERTIFICATION

Certificate of Training on Humans Subjects Protection including: Historical basis for regulating human subjects research, The Belmont Report, Scientific ethics and misconduct,

Ethical use of animals, Federal and Virginia Tech regulatory entities, and Policies and procedures; Virginia Tech, Blacksburg, VA, January 24, 2003.

Certificate of Medical Device HACCP, Association of Food and Drug Officials (AFDO), Medical HACCP Alliance course on Hazard Analysis and Critical Control Point Theory and Practical Application, Williamsburg, VA, August 20, 2003

AWARDS

Award for Technical Writing for International Graduate Students, Virginia Polytechnic Institute and State University, Division of Continued Education, English Language Institute of Virginia Tech, May 12, 2000.

CONFERENCE PROCEEDINGS & PUBLICATIONS

Presentations:

Assessment of Eye Irritation Induced by Chemicals with the Draize Test-Conference Proceeding at Student Day for Graduate Students and Post docs, “How to Present Effectively”, National Capital Area Chapter Society of Toxicology, Howard University, Washington D.C., December 12, 2002. (Potential Applications of Noninvasive Techniques to Toxicology at Cancer Center, Howard University Hospital Complex, Washington, D.C., December 12-13, 2002.)

Lindsay, D.S., **M.V. Collins**, S.M. Mitchell, R.A. Cole, G.J. Flick, J.P. Dubey. *Sporulation and survival of *Taxoplasma gondii* oocysts in artificial seawater*, Eighth International workshops on the Opportunistic Protists (IWOP-8). Hilo, Hawaii, July 25-29, 2003.

Lindsay, D.S., **M.V. Collins**, S.M. Mitchell, G.J. Flick, J.P. Dubey. Long-term survival of *Taxoplasma gondii* in Eastern Oysters (*Crassostrea virginica*). Eighth International workshops on the Opportunistic Protists (IWOP-8). Hilo, Hawaii, July 25-29, 2003.

Collins, M.V., G. Flick, D.S. Lindsay, R. Fayer, and S.A. Smith. Effects of E-Beam Irradiation on the *Cryptosporidium parvum* in Eastern Oysters (*Crassostrea virginica*). International Association for Food Protection (IAFP), New Orleans, LA, August 2003.

Collins, M.V., G. Flick, D.S. Lindsay, R. Fayer, and S.A. Smith. Effects of High Pressure Processing on infectivity of *Cryptosporidium parvum* in Eastern Oysters (*Crassostrea virginica*). High Pressure Processing Workshop and Demonstration, Blacksburg, VA, May 11-12, 2004.

Publications:

Lindsay, D.S., **M.V. Collins**, S.M. Mitchell, R.A. Cole, G.J. Flick, C.N. Wetch, J.P. Dubey. 2003. Sporulation and survival of *Toxoplasma gondii* in seawater. J. Eukaryotic Microbiology. 50(6):S687-S688.

Lindsay, D.S., **M.V. Collins**, S.M. Mitchell, G.J. Flick, J.P. Dubey, C. N. Wetch, A.C. Rosypal, A.M. Zajac, A. Lindquist. 2003. Survival of *Toxoplasma gondii* oocysts in Eastern Oysters (*Crassostrea virginica*) J. Parasitology. *In Press*.

Collins, M.V., G.J. Flick, D.S. Lindsay, R. Fayer, and S.A. Smith. 2005. Effects of E-beam and Microwave Irradiation on Eastern Oysters (*Crassostrea virginica*) experimentally infected with *Cryptosporidium parvum*. J. Parasitology. *In Review*.

Lindsay, D.S., **M.V. Collins**, G.J. Flick, C.N. Wetch, J.P. Dubey. 2005. Effects of High Pressure Processing on infectivity of *Toxoplasma gondii* oocysts in mice. J. Parasitology. *In Review*.

Collins, M.V., G.J. Flick, D.S. Lindsay, R. Fayer, and S.A. Smith. 2005. Effect of high hydrostatic pressure treatment on Eastern Oysters (*Crassostrea virginica*) experimentally infected with *Cryptosporidium parvum*. J. Parasitology. *In Review*.

Attending conferences with training courses:

*HACCP, U.S. FDA. Medical Devices Training-workshop. Roanoke, VA,
February 21-22, 2000.*

The Third International Conference in Recirculating Aquaculture, Roanoke, VA,
July 20-23, 2000.

Medical HACCP Risk Management, Training Workshop for the Medical Product Industry-
training study, Williamsburg, VA, August 20-22, 2003.

Microbes and Mucosal Immunity 2004: Careers in GI Research, Comprehensive introductory
course (NIH-sponsored program) to pursue careers in digestive health research. The Digestive
Health Center of Excellence at the University of Virginia, Charlottesville, VA, June 26-29, 2004.

LANGUAGE SKILLS

Fluent in Russian and Ukrainian.