HUMAN PROTOZOAN PARASITES IN MOLLUSCAN SHELLFISH

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I. SEAFOOD IN THE AMERICAN DIET

The popularity of seafood in the American diet is high (Munoz, 1999; Wallace *et al.*, 1999) and steadily increasing as seafood items tend to represent a healthy contribution of low-fat proteins in a balanced diet (Munoz, 1999; Rippey, 1994; Wallace *et al.*, 1999). However, some concerns have been raised worldwide regarding health risks, particularly from molluscan shellfish contaminated with human pathogens (Feldhusen, 1990; Todd *et al.*, 1992; citations in Table I).

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TABLE I

REPORTS OF ***CRYPTOSPORIDIUM*** IN MOLLUSCAN SHELLFISH INTENDED FOR HUMAN CONSUMPTION (CHRONOLOGICAL ORDER)

Reference; Shellfish species	Geographic location	Identification level; detection techniques	Comments
Fayer *et al.*, 1998; *Crassostrea virginica*	Choptank, Severn, Miles, Wye, Potomac, and Wicomico Rivers; Chesapeake Bay, USA	*Cryptosporidium parvum*; IFA, mouse bioassay	Infectious oocysts in hemolymph and gills; most infected oysters at a site near a large cattle farm
Fayer *et al.*, 1999; *C. virginica*	Fishing Bay, Tangier Sound, and Wicomico, Nanticoke, Potomac, and Patuxent Rivers; Chesapeake Bay, USA	*C. parvum* Genotype 1 and 2; IFA, mouse bioassay, PCR, PCR-RFLP	Oocysts detected in oysters and water; infectious oocysts in hemolymph and gills
Freire-Santos *et al.*, 2000; *Dosinia exoleta, Venerupis pullastra, V. rhomboideus, Venus verrucosa, Mytilus galloprovincialis, Ostrea edulis, Ruditapes philippinarum*	Galicia, northwest Spain, bounded to the Atlantic Ocean; Italy; England	*Cryptosporidium* species; malachite green, safranine, methylene blue, carbol-fuchsin, auramine-rhodamine, IFA	Depuration ineffective for *Cryptosporidium*, positive relationships between fecal coliforms and *Cryptosporidium* in shellfish
Gomez-Bautista *et al.*, 2000; *M. galloprovincialis*, *Cerastoderma edule*	Galicia, northwest Spain, bounded to the Atlantic Ocean	*C. parvum* genotype 2, IFA, mouse bioassay, PCR, RFLP	Oocysts infectious, $>10^3$ oocysts/mollusc, most contaminated shellfish near river banks with grazing cattle

Lowery *et al*., 2001; *Mytilus edulis*	Ireland, shores of Belfast Lough	*C. parvum* Genotype 1; IMS-IFA, PCR, PCR-RFLP	Anthropogenic source(s) of contamination
Fayer *et al*., 2002; *C. virginica*	Chesapeake Bay, Maryland	*Cryptosporidium* species; IFA	Oyster contamination coincided with rainfalls and increased stream flow
Gomez-Couso *et al*., 2003a; *Dosinia exoleta, Venerupis pullastra, V. rhomboideus, Venus verrucosa, Mytilus galloprovincialis, Ostrea edulis, Ruditapes philippinarum*	Galicia, northwest Spain, bounded to the Atlantic Ocean	*Cryptosporidium* species; IFA	Viable *Cryptosporidium* oocysts detected in 34% of sampled bivalves; depuration was found to be ineffective in oocyst removal
Negm, 2003; *Caelatura Iaronia pruneri*, *Donax trunculus limiacus*	Alexandria, Egypt	*Cryptosporidium* species, *Cyclospora* species, microsporidia; conventional stains, mouse bioassay	*Cryptosporidium* species oocysts and microsporidian spores were infectious; *Cyclospora* species oocysts were noninfectious
Fayer *et al*., 2003; *C. virginica*	Atlantic Coast from Maine to Florida	*C. parvum*, *C. hominis*; *C. meleagridis*, IFA, PCR, genotyping, mouse bioassay	65% commercial harvesting sites contaminated with *Cryptosporidium*
Gomez-Couso *et al*., 2004; *C. virginica*	United Kingdom	*C. parvum*, *C. hominis*; multiplexed nested PCR	*C. parvum* and *C. hominis* detected in 11% of sampled bivalves.

Since the late 1800s when shellfish-related illnesses were first reported in the United States, there have been more than 400 epidemics of food-borne diseases and more than 14,000 gastroenteritis cases related to consumption of contaminated molluscan shellfish (Rippey, 1994). New York and Florida accounted for more than 50% of these epidemics (Rippey, 1994). In New York alone from 1980 to 1994, molluscan shellfish accounted for 64% (n = 204) of all food-borne outbreaks in which the etiologic agent was identified and 41% of outbreaks caused by an unknown etiologic agent (Wallace *et al.*, 1999).

Consumed oysters are more likely than other seafood items to contain infective microorganisms because they recover and contain pathogens from surrounding waters and are very often eaten raw (Rippey, 1994; Wallace *et al.*, 1999). In the United States, 8% of approximately 33 million food-borne illnesses annually have been linked to the consumption of raw oysters (Altekruse *et al.*, 1999). Clams, mussels, cockles, and scallops are less of a public health concern because they are usually consumed cooked or steamed, which significantly alters the infectivity of potential pathogens (Rippey, 1994).

A. GASTROENTERITIS RELATED TO WASTEWATER AND SEWAGE DISPOSAL

Food-borne infections due to consumption of molluscan shellfish have been classified into three categories based on the origin of the etiologic agent (Rippey, 1994): (1) allochthonous agents (i.e., pathogens associated with sewage disposal, wastewater effluents, agricultural and urban runoff, and overboard disposal of toilet contents), (2) autochthonous agents (i.e., pathogens or marine biotoxins indigenous to coastal waters, and (3) pathogens derived from postharvest contamination due to inappropriate storage or processing. This chapter focuses specifically on allochthonous agents, that is, waterborne protozoan parasites originating from anthroponotic and zoonotic sources and found in a variety of commercial and feral molluscan shellfish species.

From 1898 to 1990, more than 75% of gastroenteritis outbreaks and more than 79% of gastroenteritis cases due to consumption of shellfish contaminated by sewage or wastewater-derived pathogens were due to an unknown etiologic agent (Rippey, 1994). In general, outbreaks and cases of gastroenteritis of unknown etiology occur more frequently in late spring and late fall, roughly coinciding with periods of the most intense feeding (i.e., filtering) and, thus, pathogen bioaccumulation (Rippey, 1994). It is believed that in more than 93% of molluscan shellfish–associated outbreaks, the shellfish were probably contaminated at the sites from which they were harvested as opposed to postharvest contamination (Wallace *et al.*, 1999).

II. BIAS IN REPORTING OF MOLLUSCAN SHELLFISH–VECTORED ILLNESSES

The data reported in medical literature most likely represent a small portion of actual gastroenteritis cases, because the true incidence of shellfish-vectored illnesses is believed to be underestimated as much as 20-fold or more (Hauschild and Bryan, 1980; Mead *et al*., 1999). There are several reasons for such underreporting. First, there is a lack of mandatory federal requirements for reporting of gastroenteritis of an unspecified nature (gastroenteritis is not a reportable illness), and physicians and state health departments are not obligated to forward case reports to federal or state authorities (Rippey, 1994; Wallace *et al*., 1999). Second, many cases of gastroenteritis are mild and self-limiting, which do not require treatment by a physician (Rippey, 1994; Wallace *et al*., 1999). Third, not all outbreaks are recognized or reported, and sporadic cases of food-borne illnesses are not detected by the existing food-borne disease surveillance system (Wallace *et al*., 1999). Fourth, it is difficult to epidemiologically ascribe a diarrhetic disease outbreak to a specific food item, particularly when small numbers of people are showing symptoms (Archer and Kvenberg, 1995; Rippey, 1994; Wallace *et al*., 1999). Fifth, for some infectious agents, symptoms may not become apparent immediately, but long after the implicated food items have been consumed or discarded (Rippey, 1994; Wallace *et al*., 1999). Finally, the accuracy of the tagging system is not perfect (Rippey, 1994).

III. ASSOCIATION OF HUMAN WATERBORNE PARASITES AND MOLLUSCAN SHELLFISH

Cryptosporidium parvum, Giardia lamblia, Cyclospora cayetanensis, and *Toxoplasma gondii* are human protozoan enteropathogens in which transmission is associated with water (Graczyk *et al*., 1997d; Lindsay, 2001; Lindsay *et al*., 2001; Ortega *et al*., 1993; Wolfe, 1992). *C. parvum*, *G. lamblia*, *C. cayetanensis* infections cause gastroenteritis, which is predominantly manifested by diarrhea (Graczyk *et al*., 1997d; Ortega *et al*., 1993; Wolfe, 1992). *T. gondii* causes serious congenital complications in fetuses born to mothers infected for the first time during pregnancy (Lindsay, 2001). Medically, the most important is *Cryptosporidium* because it significantly contributes to the mortality of people with impaired immune systems due to the lack of effective therapy (Blagburn and Soave, 1997). Although *G. lamblia* (synonyms: *Giardia intestinalis* and *Giardia duodenalis*) and *C. cayetanensis* cause serious prolonged diarrheal illness in adults and children worldwide, the infections usually respond well to pharmacological

treatment (Ortega *et al.*, 1993; Wolfe, 1992). *C. parvum, G. lamblia,* and *T. gondii* are anthropozoonotic pathogens (Graczyk *et al.*, 1997d; Lindsay, 2001; Lindsay *et al.*, 2001; Wolfe, 1992). All of these parasites produce a long-lasting and environmentally resistant infectious stage (e.g., *Cryptosporidium, Cyclospora*, and *Toxoplasma* oocysts and *Giardia* cysts, which can be transmitted via water). *Cryptosporidium* oocysts pollute coastal waters via point sources of contamination such as wastewater discharges, leaky septic tanks, urban runoff, recreational activities, and agricultural runoff predominantly from livestock operations, such as cattle farms (Graczyk *et al.*, 2000a, c). Clinical infections are mainly confined to calves, which can shed up to 10^6 oocysts/g of their feces and exceed 10^9 oocysts in daily output (Anderson, 1981). As many as 10^6 oocysts/ml can be found in human diarrhetic feces (Rose, 1997). The infectious dose of *C. parvum* for immunosuppressed people has not been established, but it is believed that the disease can be caused by a single oocyst (Rose, 1997). Mortality rates due to *C. parvum* among these individuals vary from 52 to 68% (Rose, 1997). In addition to *Cryptosporidium, Giardia, Toxoplasma,* and *Cyclospora,* human infectious microsporidia such as *Encephalitozoon intestinalis, Encephalitozoon hellem,* and *Enterocytozoon bieneusi* are emerging anthropozoonotic pathogens that inflict considerable morbidity on healthy people and can be associated with mortality in immunosuppressed populations (Bryan and Schwartz, 1999). The transmissive stages of all these parasites (i.e., oocysts, cysts, and spores) are resistant to environmental stressors and, therefore, relatively ubiquitous in the environment (Kucerova-Pospisilova *et al.*, 1999; Rose *et al.*, 1997; Wolfe, 1992). *Cryptosporidium* and *Giardia* are very frequently transmitted via water (Rose *et al.*, 1997; Wolfe, 1992). Considerable evidence indicates involvement of water in the epidemiology of microsporidia as well (Cotte *et al.*, 1999; Dowd *et al.*, 1998; Fournier *et al.*, 2000; Sparfel *et al.*, 1997).

Molluscan shellfish are suspension- or sediment-feeding organisms that filter unicellular algae, bacteria, other microorganisms, and detrital particles in the approximately 1–30 μm size range (Kennedy *et al.*, 1996; McMahon, 1991). Bivalves have an important role in aquatic habitats; by filtering suspended particles, they clarify the water and generally improve water quality (McMahon, 1991). The diameter of *Cryptosporidium, Cyclospora,* and *Toxoplasma* oocysts does not exceed 6, 8, and 10 μm, respectively, and *Giardia* cysts are oval and no longer than 15 μm (Graczyk *et al.*, 1997d; Lindsay, 2001; Lindsay *et al.*, 2001; Ortega *et al.*, 1993, Wolfe, 1992). Microsporidian spores range from 1.5 to 4 μm (Graczyk *et al.*, 2004). Thus, cystic stages of these parasites fall within the range of particles filtered by bivalve molluscs. Multiple *in vitro* and *in vivo* experimental studies demonstrated that cysts can be efficiently recovered from water and then retained and concentrated in shellfish tissue (citations in Table III).

Historically, *C. parvum* oocysts of waterborne origin were first identified in the tissue of blue mussels in Ireland (Chalmers *et al*., 1997), initiating worldwide investigation of this pathogen in molluscan shellfish (Graczyk, 2003a,b). Since then, multiple studies demonstrated that these filter-feeding organisms can harbor environmentally derived protozoan parasites as a result of concentrating the recovered particles (Graczyk, 2003b). Because of the vast amount of publications related to human protozoan parasites in molluscan shellfish, this chapter classifies available reports into three categories presented in Table I, Table II, and Table III.

An interesting epidemiological discovery is the identification, for the first time, of human infectious microsporidia spores, *E. intestinalis* and *E. bieneusi,* in molluscan shellfish; zebra mussels (*Dreissena polymorpha*) (Graczyk *et al*., 2004). Microsporidia infects a variety of vertebrate and invertebrate hosts, and approximately 14 species have been reported to infect people (Kotler and Orenstein, 1999). Of these, *E. intestinalis* and *E. bieneusi* have been reported to be zoonotic and to infect domestic animals and livestock (Bornay-Llinares *et al*., 1998; Breitenmoser *et al*., 1999; Buckholt *et al*., 2002; Deplazes *et al*., 1996; Graczyk *et al*., 2002; Rinder *et al*., 2000). In humans, they cause serious gastroenteritis, as well as urinary and sometimes ocular infections (Graczyk *et al*., 2004). Although the actual transmission route of this specific spore species is not known, it is quite possible that infectious spores of human or animal origin passed to the aquatic environments via feces or urine (Bryan and Schwartz, 1999). Spores of microsporidia have been detected in a variety of surface waters (Avery and Undeen, 1987), and water as a source of human infections has been concluded from epidemiological data (Cotte *et al*., 1999). Spores of *E. intestinalis* and *E. bieneusi* have been detected in surface waters (Dowd *et al*., 1998; Sparfel *et al*., 1997).

Cryptosporidium oocysts have also been identified in feral bivalves, supporting the concept that estuarine shellfish can be used in the sanitary assessment of water quality as biological indicators for contamination of water and sediment. Publications listed in Table II postulated the values of filter feeders as an alternative monitoring system in aquatic habitats for waterborne contamination. For example, in North America, bivalves such as zebra mussels serve as an excellent biological indicator of chemical, viral, and bacterial pollutants (e.g., in the Great Lakes and the St. Lawrence River), because they can bioaccumulate such pollutants in their tissue (Brieger and Hunter, 1993, de Lafontaine *et al*., 1999, Horgan and Mills, 1999). Zebra mussels and *Corbicula* clams very efficiently concentrate *C. parvum* and *G. lamblia* in relation to low ambient concentrations (Graczyk, 2003b). In addition, zebra mussels are also able to recover spores of human-infective species of microsporidia such as *E. intestinalis* and *E. bieneusi* (Graczyk *et al*., 2004). Bivalves such as zebra mussels or

TABLE II

REPORTS OF HUMAN WATERBORNE INTESTINAL PARASITES IN FERAL MOLLUSCAN SHELLFISH (CHRONOLOGICAL ORDER)

Shellfish species	Geographic location	Reference	Infectious agent, comments
Mytilus edulis	Ireland, Sligo area	Chalmers *et al*., 1997	*Cryptosporidium*, oocysts detected in water (22% prevalence) and mussels (8% prevalence)
Crassostrea virginica	Choptank, Severn, Miles, Wye, Potomac, and Wicomico River; Chesapeake Bay, USA	Fayer *et al*., 1998	*C. parvum*, infectious for mice, oocysts in hemolymph and gills, most infected oysters at a site near a large cattle farm
Macoma balthica, M. mitchelli	Rhode River; Chesapeake Bay, USA	Graczyk *et al*., 1999c	*Giardia duodenalis* assemblage A, on average 36 cysts/clam, contaminated clams in wildlife refuge
Ischadium recurvum	Severn and Wicomico River; Chesapeake Bay, USA	Graczyk *et al*., 1999b	*Cryptosporidium*, on average 55 oocysts/g of flesh and hemolymph homogenate
I. recurvum, C. virginica, M. balthica	Chesapeake Bay, USA	Graczyk *et al*., 2000b	*Cryptosporidium* species, *G. duodenalis* genotype (assemblage) A
Dreissena polymorpha	St. Lawrence River near Sainte-Foy; Quebec, Canada	Graczyk *et al*., 2001	*C. parvum* genotype 1, anthropogenic source, oocysts in hemolymph and flesh, 4.4×10^2 oocysts/mussel
D. polymorpha	Shannon River, Ireland	Graczyk *et al*., 2004	*C. parvum, G. lamblia, Encephalitozoon intestinalis, Enterocytozoon bieneusi*. Viable parasites identified by fluorescence *in situ* hybridization (FISH) method.

Corbicula clams are convenient for such purposes because they form dense populations and clusters, which facilitate the collection of large samples, do not have economic value, have a relatively small size, and are easily collected throughout the year (McMahon, 1991).

A. QUANTITATIVE ESTIMATION OF REMOVAL OF HUMAN WATERBORNE PARASITES

Zebra mussels collected from the St. Lawrence River, Canada, near a wastewater discharge site contained on average approximately 440 *C. parvum* oocysts/mussel (Graczyk *et al.*, 2001). Knowing the *C. parvum* retention rate as 4.9×10^2 oocysts/mussel/24 hr (Frischer *et al.*, 1999) and *D. polymorpha* densities of approximately 3.0×10^4 specimens/m^2 for adult (>1 year old) mussels (McMahon, 1991), it has been calculated that during 24 hours, approximately 1.3×10^7 waterborne *C. parvum* oocysts can be removed by each square meter of mussel bed in the St. Lawrence River (Graczyk *et al.*, 2001).

The concentration of *C. parvum* observed in zebra mussels from the Shannon River, Ireland (Graczyk *et al.*, 2004), was much lower than that reported from the St. Lawrence River (Graczyk *et al.*, 2001). However, in the St. Lawrence River, mussels originated from sites affected by wastewater discharge, and in the Shannon River, no apparent sources of water contamination have been identified near any of the sites. Considering the natural densities of zebra mussels (McMahon, 1991), and the fact that on average approximately eight parasites per mussel have been identified in the Shannon River study (Graczyk *et al.*, 2004), at least 2.4×10^5 pathogens/24 hr can be potentially removed per square meter of zebra mussel bed in the Shannon River.

B. CAN HUMAN PARASITES INFECT MOLLUSCAN SHELLFISH?

A single report suggests indigenous infection of *Cryptosporidium* in bivalves (Azevedo, 1989). Organisms supposedly resembling *Cryptosporidium* species merozoites were found in the gill epithelium of Portuguese clams (*Ruditapes decussatus*); however, as no other stages were observed, this finding was considered by the author as inconclusive (Azevedo, 1989).

C. A PUBLIC HEALTH THREAT FROM MOLLUSCAN SHELLFISH CONTAMINATED WITH *CRYPTOSPORIDIUM*

Before 1992, the association between contamination derived from animal fecal wastes and the occurrence of shellfish-vectored illnesses was inconclusive (Stelma and McCabe, 1992). In 1994, enterohemorrhagic *Escherichia*

TABLE III

EXPERIMENTAL EXPOSURE OF MOLLUSCAN SHELLFISH TO TRANSMISSIBLE STAGES OF HUMAN INTESTINAL WATERBORNE PARASITES (CHRONOLOGICAL ORDER)

Shellfish species	Reference	Parasite species	Comments
Crassostrea virginica	Fayer *et al.*, 1997	*Cryptosporidium parvum*	*In vivo* retention, infectious oocysts in hemocytes, gills, and gut, oocyst after 1 wk in oysters
Corbicula fluminea	Graczyk *et al.*, 1997c	*C. parvum*	*In vitro* phagocytosis, 82% of 1.1×10^5 oocysts phagocytosed after 2 hr
C. fluminea	Graczyk *et al.*, 1997a	*Giardia duodenalis*	*In vitro* phagocytosis, 86% of hemocytes showed phagocytosis after 2 hr exposure
C. virginica	Graczyk *et al.*, 1997b	*C. parvum*	*In vitro* phagocytosis, 95% of hemocytes showed phagocytosis after 2 hr exposure
C. fluminea	Graczyk *et al.*, 1998a	*Cyclospora cayetanensis*	Retention rate: 4.6×10^2 oocysts/clam/24 hr
C. fluminea	Graczyk *et al.*, 1998b	*C. parvum*	Retention rate: 1.9×10^5 oocysts/clam/24 hr
C. virginica	Graczyk *et al.*, 1998c	*C. parvum*, *G. duodenalis*	Specific detection of *C. parvum* and *G. duodenalis* in tissue of oysters carrying oyster diseases
C. fluminea	Graczyk *et al.*, 1999a	*G. duodenalis*	Retention rate: 1.3×10^3 cysts/clam/24 hr

Mytilus edulis	Graczyk *et al.*, 1999b	*C. parvum*	Detection threshold: 19 oocysts/g spiked-to-recovered oocysts: 51%
M. galloprovincialis	Tamburrini and Pozio, 1999	*C. parvum*	Retention rate: 1×10^7 oocysts/mussel/24 hr, infectious oocysts in hemocytes, gills, and gut, oocyst after 2 wks in oysters
Ruditapes philippinarum	Freire-Santos *et al.*, 2001	*C. parvum*	*In vivo* retention, oocysts in gills and gut, oocyst infectious after 48 hr in clams
C. virginica	Lindsay *et al.*, 2001	*Toxoplasma gondii*	*T. gondii* oocysts can be removed from water by oyster and retain their infectivity
Ostrea edulis, Tapes decussatus	Freire-Santos *et al.*, 2002	*C. parvum*	Infectivity of *C. parvum* oocysts demonstrated at 31 days after contamination
Mytilus galloprovincialis	Arkush *et al.*, 2003	*T. gondii*	*T. gondii* oocysts retain infectivity for at least 3 days in bivalve tissue
Dreissena polymorpha, C. fluminea	Graczyk *et al.*, 2003	*C. parvum, Giardia lamblia*	*D. polymorpha* and *C. fluminea* can accumulate human waterborne parasites in proportion to ambient concentrations
C. virginica	Lee and Lee, 2003	*Eimeria acervulina*	Oocysts retained in oyster tissue no longer than 96 hr after contamination
Corbicula japonica	Izumi *et al.*, 2004	*C. parvum*	Single exposure to pathogen results in a rapid intake and secretion of viable oocysts

coli O157 became of major concern (Rippey, 1994). This bacterium has not been associated with shellfish; however, its frequent occurrence in cattle indicated potential public health problems with shellfish harvested from waters affected by runoff from cattle farms (Rippey, 1994).

Beginning in 1998, multiple studies worldwide indicated that molluscan shellfish intended for human consumption can be contaminated with *Cryptosporidium* (citations in Table I). So far there has been no reported outbreak (or case) of food-borne cryptosporidiosis linked to consumption of raw oysters in the United States. However, more than 40% of all food-borne infections linked to oyster consumption are in the category of an unknown etiologic agent (Anonymous, 1996). In addition, 20% of the general U.S. population are vulnerable to *C. parvum* infection (Gerba *et al.*, 1996), the epidemiology of enteric infections (i.e., cryptosporidiosis) indicates an association with consumption of raw shellfish (Fang *et al.*, 1991), and it is believed that in the United States and Canada, the true incidence of shellfish-vectored gastroenteritis is considerably underestimated (Hauschild and Bryan, 1980). As stated earlier in this chapter, there is no mandatory federal requirement for reporting of gastroenteritis of an unspecified nature and physicians, and state health departments are forwarding case reports to federal authorities (Rippey, 1994; Wallace *et al.*, 1999).

D. GLOBAL PROBLEM

Protozoan parasite contamination of molluscan shellfish destined for human consumption is not limited to North America, and it appears as a global problem. Such contamination has been reported from Spain (Freire-Santos *et al.*, 2000; Gomez-Bautista *et al.*, 2000; Gomez-Couso *et al.*, 2003a), United Kingdom (Freire-Santos *et al.*, 2000; Gomez-Couso *et al.*, 2004; Lowery *et al.*, 2001), Italy (Freire-Santos *et al.*, 2000), and Egypt (Negm, 2003). More importantly, in some intensive seafood production regions such as northwest Gallicia, Spain, where molluscan shellfish production is the most important industry, cases of self-limiting diarrhea associated with consumption of raw oysters and clams are often reported (Freire-Santos *et al.*, 2001). Also, molluscan shellfish consumed raw are seriously considered a food vehicle for *Cryptosporidium* transmission in Switzerland (Baumgartner *et al.*, 2000).

E. POTENTIAL METHODS OF SANITIZATION

Molluscan shellfish destined for human consumption can be subjected to processing such as depuration, irradiation, ozonation, and high-pressure processing in order to remove or inactivate potential microbiological

contaminants. These methods have been applied predominantly to purge or inactivate bacterial and viral agents, and the published information on their efficiency for protozoan parasites is limited. Gomez-Couso *et al*. (2003a) demonstrated that depuration was ineffective in removing *C. parvum* oocysts from mussels, oysters, clams, and cockles harvested from contaminated waters. This laboratory also demonstrated that molluscan shellfish contaminated with *C. parvum* oocysts can spread this contamination within the commercial depuration plants to other aquatic organisms processed in such facilities (Gomez-Couso *et al*., 2003b).

IV. METHODS USED FOR IDENTIFICATION OF HUMAN PROTOZOAN PARASITES IN MOLLUSCAN SHELLFISH

Methods for identification of human protozoan parasites in the tissue of molluscan shellfish include (1) immunofluorescent antibodies (IFA) alone or in combination with immunomagnetic separation (IMS), (2) polymerase chain reaction (PCR) alone or combined with restricted fragment length polymorphism (RFLP) for genotyping, (3) multiplexed nested PCR, and (4) fluorescence *in situ* hybridization (FISH). Infectivity of the parasites recovered from the shellfish are usually assessed by mouse bioassays.

Because *Cryptosporidium, Giardia,* and microsporidia can infect a variety of nonhuman hosts (Graczyk *et al*., 1997; Kotler and Orenstein, 1999; Wolfe, 1992), identification of human infectious species is a challenge. Another challenge is determination of the viability of these environmentally recovered pathogens, because they may be nonviable and, thus, not of epidemiological importance. Although molecular methods are very sensitive and specific, they do not assess infectivity of the pathogens recovered from shellfish. Both challenges are met by the FISH technique. FISH employs fluorescently labeled oligonucleotide probes targeted to species-specific sequences of 18S ribosomal RNA (rRNA), and therefore, identification of pathogens is species specific (Graczyk *et al*., 2002, 2004; Hester *et al*., 2000). Also, because rRNA has a short half-life and is present only in numerous copies in viable organisms, FISH allows for differentiation between viable and nonviable pathogens (Dorsch and Veal, 2001; Graczyk *et al*., 2002; Hester *et al*., 2000; Jenkins *et al*., 2003; Vesey *et al*., 1998). FISH has been combined with direct IFA against the wall antigens of *Cryptosporidium* and *Giardia,* and this approach has been successful for detection of *C. parvum* and *G. lamblia* in shellfish samples (Graczyk *et al*., 2004). For identification of viable pathogens such as *C. parvum, G. lamblia,* or human-infective microsporidia, FISH is advantageous over other techniques including PCR because it allows simultaneous species-specific identification, visualization,

and viability assessment of single pathogen cells. Such resolution is not available or is extremely impractical with any other technique. For example, using highly sensitive RT-PCR, the lowest number of *C. parvum* oocysts that can be assessed for viability is 10^3 (Jenkins *et al.*, 2000).

V. WHY ARE THE SHELLFISH CONSUMPTION–CAUSED ILLNESSES NOT ANTICIPATED TO DECLINE?

There are several reasons that shellfish-vectored outbreaks and cases of gastroenteritis are not projected to decline and *Cryptosporidium* is being identified in bivalve molluscs intended for human consumption.

1. The fecal coliform count, which is the main standard indicator for waterborne fecal contamination, is not reliable in determining the quality of water at shellfish-harvesting sites (Anonymous, 1996; Rippey, 1994; Wilson and Moore 1996). The transmissive stages of human protozoan parasites can persist in aquatic environments for a longer time as compared to enteric and indicator bacteria (Graczyk and Schwab, 2000; Power and Collins, 1989; Richards, 1988). Although *C. parvum* oocysts are excreted with feces, waterborne *Cryptosporidium* pollution does not correlate with fecal coliform counts (Graczyk *et al.*, 2000c; Rose, 1997). Thus, waters considered to be "safe" based on the fecal coliform standards may, in fact, be contaminated by human enteric parasites (Anonymous, 1996; Graczyk and Schwab, 2000; Rippey, 1994; Wallace *et al.*, 1999).
2. Animal operations such as individual farms or huge industrial animal production facilities (e.g., beef and dairy cattle) located near shores can generate enormous surface runoff, particularly under adverse weather conditions, and can cause water pollution (Fayer *et al.*, 1998, 1999; Freire-Santos *et al.*, 2000; Gomez-Bautista *et al.*, 2000).
3. Deficiencies at the sewage treatment plants such as volume limitations related to designed capacity of a plant under adverse weather conditions such as heavy rainfall allow the discharge of large amounts of unprocessed waste waters. In addition, the periodic breakdown in particle removal or inadequate disinfection can deliver human enteropathogens into shellfish-harvested waters (Rippey, 1994).
4. Transmissive stages of human enteric parasites are resistant to environmental degradation (heat, sunlight, temperature fluctuations, etc.) and may even remain infectious after exposure to chemical water treatment processes such as chlorination (Graczyk and Schwab, 2000; McDonnell *et al.*, 1997). These pathogens can still be infectious even after the oyster meat has been processed (Graczyk and Schwab, 2000; McDonnell *et al.*, 1997) and are very poorly (i.e., slowly) depurated (removed) from molluscan

shellfish tissue (Power and Collins, 1989; Richards, 1988; Schwab *et al.*, 1998).

5. Increased fecal pollution determined on the fecal coliform counts has decreased the total area of coastal habitats approved for harvesting of molluscan shellfish, particularly oysters, for human consumption (Rippey, 1994). Thus, large and very productive areas have been closed, resulting in illegal harvesting of oysters from unapproved or closed but profitable waters (Rippey, 1994). Such criminal activity unavoidably affects public health when contaminated shellfish enter the market (Rippey, 1994).

6. Improper postharvest handling and transportation of molluscan shellfish (i.e., temperature abuse) affect oysters directed for consumption in a raw form (Rippey, 1994). Holding of oysters at temperatures higher than 4°C in transit or in the market place can contribute to multiplication of bacterial enteropathogens (Rippey, 1994).

7. Many shellfish-related outbreaks have more than one contributing factor (Wallace *et al.*, 1999). For example, contaminated ingredients added to raw or lightly cooked molluscs have been also reported as contributing factors for food-borne infections (Wallace *et al.*, 1999).

8. Development of new molecular techniques that can be applied to a wide variety of food items has dramatically increased the sensitivity and specificity of detection of human enteric parasites in the tissue of molluscan shellfish (Schwab *et al.*, 1998, 2000; citations in Table I).

VI. CONCLUSIONS

1. Food-borne illnesses following consumption of molluscan shellfish continue to occur despite that (1) testing of waters for fecal coliforms from which oysters are harvested for human consumption demonstrate that the water quality met the criteria of the National Shellfish Sanitation Program (NSSP), (2) oysters harvested from the NSSP-approved waters are considered "safe" with regard to fecal pollution, (3) sanitation procedures at oyster-harvesting facilities met standards set by the state authorities, and (4) in most instances, neither confirmed evidences of improper handling or processing of outbreak-implicated oysters nor the environmental sources of pollution were detected. These facts indicate that the monitoring of water for fecal coliform at molluscan shellfish-harvesting sites may not be sufficient indicating the presence of human waterborne parasites such as *Cryptosporidium, Cyclospora, Giardia, Toxoplasma,* and human infectious microsporidia (Fayer *et al.*, 1998; Graczyk and Schwab, 2000; Rippey, 1994; Wallace *et al.*, 1999).

2. Oocysts of *C. parvum* can retain their infectivity in molluscan shellfish (Fayer *et al.*, 1997; Freire-Santos *et al.*, 2001; Tamburrini and Pozio, 1999), and standard depuration processes applied to commercially harvested bivalve molluscs are not effective for *Cryptosporidium* (Freire-Santos *et al.*, 2000, 2001; Gomez-Bautista *et al.*, 2000).

3. Reducing the number of outbreaks of food-borne diseases due to bivalve molluscs will require the coordinated efforts of different agencies involved in water quality assessment, shellfish harvesting and processing, disease surveillance, and consumer education (Anonymous, 1996; Rippey, 1994; Wallace *et al.*, 1999). It may be useful to reduce or eliminate economic incentives for illegal harvesting of shellfish from unapproved or prohibited waters that results in contaminated shellfish reaching the market place (Rippey, 1994).

4. Estuarine molluscan shellfish, both commercial and feral, can be used for the sanitary assessment of water quality as biological indicators for water and sediment contamination with *Cryptosporidium, Giardia, Toxoplasma,* and *Cyclospora* (Xiao *et al.*, 1998, 2001; citations in Tables I and II).

5. Prevention of food-borne gastroenteritis due to consumption of molluscan shellfish relies on consumer education and thorough cooking of shellfish (Fayer *et al.*, 1998, 1999; Freire-Santos *et al.*, 2001; Rippey, 1994; Wallace *et al.*, 1999). Education should be particularly focused on populations that are predisposed to serious illness after consumption of contaminated shellfish (e.g., people with suppressed immune systems) (Fayer *et al.*, 1999; Rippey, 1994; Wallace *et al.*, 1999). Several factors may impede consumer education campaigns about the risk of raw shellfish consumption (Altekruse *et al.*, 1999).

6. It may be useful to institute guidelines for processing steps to reduce pathogen counts in molluscan shellfish (e.g., cold shock, heat shock, pasteurization, or irradiation) (Altekruse *et al.*, 1999). Because oocysts of *C. parvum* can be rendered noninfectious by heating to temperatures higher than 72°C, it has been recommended that shellfish be cooked before eating, particularly for persons with any type of immune deficiency (Fayer *et al.*, 1998, 1999; Freire-Santos *et al.*, 2001).

7. Continued surveillance for outbreaks and cases of gastroenteritis associated with consumption of raw shellfish are needed to assess the efficacy of the NSSP in preventing human illnesses (Graczyk and Schwab, 2000; Rippey, 1994; Wallace *et al.*, 1999). Public health officials should consider consumption of raw shellfish as a possible source of infection during the evaluation of a gastroenteritis outbreak (Graczyk and Schwab, 2000; Rippey, 1994; Wallace *et al.*, 1999).

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